ORP5 TRANSFERS PHOSPHATIDYLSERINE TO MITOCHONDRIA AND REGULATES MITOCHONDRIAL CALCIUM UPTAKE AT ENDOPLASMIC RETICULUM - MITOCHONDRIA CONTACT SITES

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ABSTRACT

Mitochondria are dynamic organelles essential for cell survival whose structural and functional integrity rely on selective and regulated transport of lipids from/to the endoplasmic reticulum (ER) and across the two mitochondrial membranes. As they are not connected by vesicle transport, the exchange of lipids between ER and mitochondria occurs at sites of close organelle apposition called membrane contact sites. However, the mechanisms and proteins involved in these processes are only beginning to emerge. Here, we show that ORP5/8 mediate non-vesicular transport of Phosphatidylserine (PS) from the ER to mitochondria in mammalian cells. We also show that ER-mitochondria contacts where ORP5/8 reside are physically and functionally linked to the MIB/MICOS complexes that bridge the mitochondria membranes, cooperating with them to facilitate PS transfer from the ER to the mitochondria. Finally, we show that ORP5 but not ORP8, additionally regulates import of calcium to mitochondria and consequently cell senescence.

KEYWORDS

Membrane contact sites, mitochondria, ORP, lipid transfer, calcium

ABBREVIATIONS

APOO: Apolipoprotein O

APOOL: Apolipoprotein O-like

CHCHD3/6: Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 3/6

Calcium: Ca^{2+}

CCCP: Carbonyl cyanide 3-chlorophenylhydrazone

CJ: cristae junctions

CV-ATP5A: complex V alpha subunit

EM: electron microscopy

ER: endoplasmic reticulum

FFAT: two phenylalanines (FF) in an Acidic Tract

GRP75: glucose-regulated protein 75

HRP: horseradish peroxidase

IF: immunofluorescence

IMM: inner mitochondrial membrane

IMS: intermembrane space

IP3R: Inositol trisphosphate receptor

LAR-GECO: low-affinity red fluorescent genetically encoded Ca2+ indicators for optical imaging

LTPs: lipid transfer proteins

MAM: mitochondria-associated ER membrane

MCU: mitochondrial calcium uniporter

MIB: Mitochondrial intermembrane space bridging complex

MICOS: Mitochondrial Contact Sites and Cristae Junction Organizing System

MINOS1: mitochondrial inner membrane organizing system 1

76 MS: mass spectrometry

77 mt-Pericam: mitochondrial permuted YFP and a CaM (calmodulin)

78 OMM: outer mitochondrial membrane

79 ORD: OSBP-related lipid-binding/transfer domain

80 ORP: Oxysterol binding protein (OSBP)-related proteins

81 Osh: oxysterol-binding homology

82 PC: Phosphatidylcholine

83 PDI: Protein disulfide isomerase

84 PE: phosphatidylethanolamine

85 PH: pleckstrin homology

86 PI4P: Phosphatidylinositol-4-phosphate

87 PIP2: Phosphatidylinositol 4,5-bisphosphate

88 POPC: Palmitoyloleoylphosphatidylcholine

89 PS: phosphatidylserine

90 PTPIP51: Protein tyrosine phosphatase interacting protein 51

91 QIL1: MICOS complex subunit MIC13

92 R-GECO: Red fluorescent, genetically-encoded Ca2+ indicator for optical imaging

93 SA β-gal: Senescence-associated β –galactosidase

94 SAM: Sorting and Assembly Machinery

95 SAM50: Sorting Assembly Machinery 50

96 TLC: thin layer chromatography

97 TMRE: Tetramethylrhodamine ethyl ester

98 VAPB: Vesicle-associated membrane protein-associated protein B

99 VDAC: Voltage Dependent Anion Channel

100 WB: western blotting

INTRODUCTION

Vesicular trafficking is the major pathway for transport of proteins and metabolites, such as lipids, between membranes. However, an alternative route, which is vesicle-independent, occurs at regions of close inter-organelle membrane proximity (within less than 30 nm) also called membrane contact sites (Scorrano, De Matteis et al., 2019). This route is particularly important to preserve membrane composition, integrity and identity of the intracellular organelles such as the mitochondria that are largely excluded from the classical vesicle-mediated trafficking pathways. Like other organelles, mitochondria can be closely associated with the endoplasmic reticulum (ER), the major site of lipid synthesis and the major intracellular calcium (Ca^{2+}) store. ER membrane subdomains closely apposed to the mitochondria are called mitochondria-associated ER membranes (MAMs) and they facilitate the exchange of Ca^{2+} and lipids between the two organelles (Herrera-Cruz & Simmen, 2017, Tatsuta, Scharwey et al., 2014, Vance, 2014)

Mitochondria are involved in a plethora of cellular processes including energy production, lipid metabolism, Ca^{2+} homeostasis and apoptosis. To fulfill their numerous functions, mitochondria need to maintain a defined membrane composition by receiving essential lipids and lipid precursors from the ER through membrane contact sites (Giordano, 2018, Vance & Tasseva, 2013).

Increasing lines of evidence suggest that lipid transfer proteins (LTPs) play a major role in regulating the lipid composition of membranous organelles by facilitating non-vesicular lipid transport at membrane contact sites. In recent years, several tethering complexes with lipid transfer activity have been identified at membrane contact sites between the ER and other intracellular organelles including the plasma membrane (PM) in yeast as well as in mammalian cells. However, the LTPs of ER-mitochondria

membrane contact sites remain still mysterious. The best-studied lipid transfer/tethering complex at ER-mitochondria contact sites is the yeast ER-mitochondria encounter structure (ERMES) (Kornmann et al 2009, Lang et al 2015) that bridges the ER and the mitochondrial membranes and also facilitates the exchange of phospholipids (in particular phosphatidylcoline, PC) between them. Despite its important functions in yeast, the ERMES complex is not conserved in metazoans. In metazoans, very little is known on how lipids are exchanged at ER-mitochondria membrane contact sites and about the proteins involved in this process. Some tethers at mammalian ER-mitochondria contact sites have started to emerge in the past years (Gatta and Levine 2017), including the Mitochondrial Ca^{2+} Uniporter (MCU)–Voltage Dependent Anion Channel (VDAC)–Glucose Regulated Protein 75 (GRP75)–Inositol Triphosphate Receptor (IP3R) machinery involved in Ca^{2+} import from the ER to mitochondria (Rizzuto et al. 2009) and the recently identified Protein tyrosine phosphatase interacting protein 51 (PTPIP51)–Vesicle-associated membrane protein-associated protein B (VAPB) complex (Stoica et al 2014) alteration of which induces defects in ER-mitochondria tethering and Ca^{2+} exchanges. However, none of these proteins has been directly involved in non-vesicular lipid transport between ER and mitochondria membranes.

The Oxysterol binding protein (OSBP)-related proteins constitute a large family of LTPs conserved from yeast (Osh) to humans (ORP) and localized to different subcellular sites, shown in several cases to be membrane contact sites. A common feature of all ORPs is the presence of an OSBP-related lipid-binding/transfer (ORD) domain. Most ORP proteins contain an FFAT domain that binds ER-localized VAP proteins and a pleckstrin homology (PH) domain that interacts with lipids or proteins in distinct non-ER organelle membranes. Two members of this family, ORP5 and

151 ORP8, do not contain a FFAT domain but are directly anchored to the ER through
152 their C-terminal domain. We have recently shown that ORP5 and ORP8 localize to
153 ER-mitochondria contact sites where they play a key role in maintaining
154 mitochondrial morphology and respiratory function (Galmes et al. 2016). ORP5 and
155 ORP8 have been previously shown to transfer phosphatidylserine (PS) from the
156 cortical ER to the PM, in counter-exchange with the phosphoinositides
157 Phosphatidylinositol-4-phosphate (PI4P) and Phosphatidylinositol 4,5-bisphosphate
158 (PIP_2) (Chung et al. 2015; Ghai et al, 2017). Interestingly, transport of PS occurs also
159 at ER-mitochondria contact sites. Newly synthesized PS, by the ER-localized PS-
160 Synthase 1 (PSS1), is shuttled from the ER to the outer mitochondrial membrane
161 (OMM) and from OMM to inner mitochondrial membrane (IMM) where it is rapidly
162 converted to phosphatidylethanolamine (PE) (Vance, 1990, Vance & Tasseva, 2013). At
163 the IMM, PE plays crucial roles in maintaining mitochondrial tubular morphology and
164 therefore in mitochondrial respiratory functions (Joshi, Thompson et al., 2012,
165 Steenbergen, Nanowski et al., 2005). Regardless of extensive studies on PS transport
166 between ER and mitochondria since its first discovery more than 20 years ago (Vance,
167 1990), the mechanisms underlying this transport and the proteins involved are still
168 elusive.

169 Here we show that ORP5 and ORP8 are the LTPs that transfer PS from the ER
170 to the mitochondria at ER-mitochondria contact sites in mammalian cells. We also
171 show that they are physically and functionally connected with Sorting Assembly
172 Machinery 50 (SAM50) and Mitofilin, two components of the Mitochondrial
173 Intermembrane space Bridging (MIB) and Mitochondrial Contact sites and Cristae
174 junction Organizing System (MICOS) complexes, that anchors the IMM to the OMM.
175 The MICOS complex (composed of seven proteins: Mitofilin, CHCHD3, CHCHD6,

176 APOO, QIL1, APOOL and MINOS1) is preferentially located at Cristae Junctions (CJ),
177 tubular structures that connect the IMM to the cristae, and it is necessary for CJ
178 formation, cristae morphology and mitochondria function (Harner, Korner et al., 2011,
179 Huynen, Muhlmeister et al., 2016, Ott, Dorsch et al., 2015, Wollweber, von der
180 Malsburg et al., 2017). The integral IMM protein Mitofilin is the central component of
181 the MICOS complex and carries a large domain exposed to the mitochondria
182 intermembrane space (IMS) that interacts with the OMM Sorting and Assembly
183 Machinery (SAM) to form the MIB complex (Friedman, Mourier et al., 2015, Guarani,
184 McNeill et al., 2015). The SAM complex is constituted of SAM50 (a pore-forming β-
185 barrel protein), Metaxin1 and 2, and is involved in the membrane insertion and
186 assembly of mitochondrial β-barrel proteins (Hohr, Lindau et al., 2018, Kozjak,
187 Wiedemann et al., 2003, Kozjak-Pavlovic, Ross et al., 2007) (see cartoon in Figure
188 8).

189 Here we show that ORP5/8 interact and cooperate with SAM50 and Mitofilin to
190 facilitate the movement of PS from the ER to the mitochondria across ER-
191 mitochondria contact sites and the IMS and consequently the synthesis of
192 mitochondrial PE. Finally, we uncover an additional role for ORP5, but not ORP8, in
193 regulating Ca^{2+} import to mitochondria, through regulation of SAM50-VDAC protein
194 levels. Our findings reveal a novel link between lipid and Ca^{2+} transport at ER-
195 mitochondria contact sites and provide a mechanistic insight of the specific role of
196 ORP5 in modulating cellular senescence.
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200

201 **RESULTS**

202 **ORP5 and ORP8 transport PS from the ER to the mitochondria at ER-**
203 **mitochondria contact sites**

204 We have recently reported that ORP5 and ORP8, shown to transfer lipids at ER-PM
205 contacts, localize also to ER-mitochondria contact sites (Galmes, Houcine et al., 2016).
206 However, their role in lipid transport at ER-mitochondria contacts still remains to be
207 established. Taking into account their role in counter-exchanging PS with the PM
208 phosphoinositides PI4P and PIP_2 at ER-PM contacts (Chung et al. 2015; Ghai et al,
209 2017), we hypothesized that ORP5 and ORP8 could also mediate PS transport at ER-
210 mitochondria contact sites.

211 The ORD domain of ORP8 has been shown to transfer PS (in counter-transport
212 with PI4P or PIP_2) between liposome membranes *in vitro* (Chung et al. 2015; Ghai et
213 al, 2017). Since PI4P and PIP_2 are not present on mitochondrial membranes it is
214 possible that at ER-mitochondria contact sites ORP5/8 might transport back to the
215 ER other phospholipids, such as PE. However, no study so far has ever addressed
216 the ability of ORP5 and ORP8 ORD domains (ORD5, ORD8) to transfer other
217 phospholipids between liposomes *in vitro.* First, to compare and study the role of
218 ORD5 and ORD8 in the transfer of phospholipids *in vitro*, we purified the recombinant
219 ORD5 (aa 265-703) and ORD8 (aa 328-767) in bacteria (Escherichia coli) (Fig. S1a)
220 and analyzed their lipid transfer ability by measuring the transport of fluorescent
221 phospholipids (TopFluor-PS, -PC or -PE) from donor to acceptor liposomes *in vitro*.
222 Donor liposomes containing fluorescent phospholipids and biotinylated lipids
223 (liposomes composed of 1 mol% TopFluor-PS, -PC or -PE and 2 mol% of
224 biotinylated-PE, and 97 mol% POPC) were first immobilized on streptavidin beads
225 and then mixed with acceptor liposomes (composed of 100 mol % POPC) in

226 presence or absence of ORP5/8 ORD domains (Fig. 1a). After 1 hour at 37°C,
227 acceptor liposomes were recovered from the supernatant and their fluorescence was
228 measured (Fig. 1a). Our results show that both ORD5 and ORD8 transfer PS, but not
229 PC and PE, from donor to acceptor liposomes (Fig. 1b). They also reveal that ORD5
230 and ORD8 share equivalent abilities to transfer PS *in vitro*.

231 Next, we addressed whether ORP5 and ORP8 could mediate non-vesicular
232 transfer of PS at ER-mitochondria contact sites *in situ*. As the ER-derived PS is the
233 major precursor for mitochondrial PE, if ORP5 and ORP8 mediate non-vesicular
234 transport of PS from the ER to the mitochondria, then their absence should lead to a
235 reduction of mitochondrial PE. To test this, we used Percoll gradient-based
236 subcellular fractionation (Galmes et al. 2016) to isolate pure mitochondria from HeLa
237 cells where ORP5 or ORP8 were transiently silenced by RNAi. We chose to use a
238 transient knockdown as it overcomes the limits and/or compensatory effects on lipid
239 transport/biosynthetic pathways that other stable approaches could induce. The
240 purity of mitochondria and of the other subcellular fractions was verified in control,
241 ORP5 and ORP8 knockdown conditions by western blotting (WB) (Fig. S1b). As
242 controls for the purity of subcellular fractions, the samples were probed for
243 cytochrome c as mitochondrial marker and IP3R-3 as a MAM-enriched marker. All
244 markers were highly enriched in their respective fractions and were absent in the
245 others. In accord with our previous study (Galmes et al., 2016), ORP5 and ORP8
246 were enriched in the MAM fraction and absent in the mitochondria fraction of control
247 cells. On the contrary, they were strongly suppressed in ORP5 and ORP8
248 knockdown cell lysates and in the respective MAM fractions (Fig. S1b). The PE
249 content of purified mitochondria from ORP5 and ORP8 knockdown was then
250 analyzed by mass spectrometry (MS)-based lipidomics, revealing a reduction of PE

levels in mitochondria isolated from ORP5 and ORP8 knockdown cells of 34% and 20%, respectively, as compared to control cells (Fig. 1c). Interestingly, PE levels of the total cells were unchanged, indicating a specific effect of ORP5 or ORP8 depletion on mitochondrial PE.

In subsequent experiments, we measured the levels of mitochondrial PE newly synthesized from the ER-derived PS by using a radiometric PS-to-PE conversion assay *in situ* (Shiao, Lupo et al., 1995) in silenced or control HeLa cells (Fig. S1c-1d). This assay allows the monitoring of PS transfer from the ER to mitochondria by measuring the levels of radioactive PS and PS-derived PE by thin layer chromatography (TLC) after 18h of incorporation of radioactive L-[^{3}H(G)]-serine into the cells. A significant decrease in the levels of newly synthetized PE was found in ORP5 knockdown and in ORP5+ORP8 double-knockdown cells (Fig. 1d). The decrease was stronger in ORP5+ORP8 double-knockdown cells, indicating a cooperative effect of ORP5 and ORP8 in this process. A slight decrease was also found in ORP8 knockdown cells, although not statistically significant, suggesting a major role of ORP5 compared to ORP8 in PS transfer at ER-mitochondria contact sites *in situ*.

Even a modest reduction (22-27%) of mitochondrial PE levels in mammalian cells have been shown to profoundly alter the morphology of mitochondrial cristae as well as mitochondria functions (Tasseva, Bai et al., 2013). Thus, the decrease in mitochondrial PE in ORP5 and ORP8 knockdowns is in accord with our previous electron microscopy (EM) observations that knockdown of ORP5 or ORP8 alters the architecture of the cristae, while ER-mitochondria contact sites with normal morphology were still visible (Galmes et al., 2016). However, our previous study did not rule out that these contact sites could be affected in their number/size. To test

276 whether the effects on PS transport at ER-mitochondria contacts were specific of
277 ORP5 and ORP8 loss of function or simply due to a decrease of ER-mitochondria
278 contacts induced by their knockdown, we quantified the abundance of ER-
279 mitochondria contact sites by EM in control and ORP5, ORP8, ORP5+ORP8
280 knockdown cells. To facilitate the visualization of the ER we transfected the cells with
281 a HRP-KDEL construct (carrying a horseradish peroxidase (HRP) tagged with an ER
282 retention motif) that stains the ER with a dark signal. Our quantifications revealed
283 that ORP5, ORP8 or ORP5+ORP8 knockdowns did not affect the extent of ER-
284 mitochondria contact sites (Fig. 1e-1f). These results indicate that ORP5 and ORP8
285 silencing affect non-vesicular PS transport at ER-mitochondria contacts but not their
286 extent and integrity, confirming that at these sites ORP5 and ORP8 act exclusively as
287 LTPs and not as tether (Fig. 1h). Additionally, 52% of mitochondria in ORP5+ORP8
288 double-knockdown cells display aberrant cristae morphology versus 9% in control
289 cells (Fig. 1e-1g). These defects in cristae morphology were stronger than what we
290 had previously shown in the case of ORP5 and ORP8 individual knockdowns
291 (Galmes et al., 2016), and most likely reflect the stronger effect of ORP5+ORP8
292 double-knockdown on PS transport at ER-mitochondria contact sites.
293
294 **ER-mitochondria contact sites marked by ORP5/8 are physically connected**
295 **to mitochondrial membranes via ORP5/8 interaction with the mitochondrial**
296 **intermembrane space bridging (MIB) complex**
297 To investigate whether ORP5/8 localize to specific ER-mitochondria contact site
298 subdomains we performed a morphological analysis of ORP5 localization by
299 immuno-EM (IEM) on ultrathin cryosections from HeLa cells transfected with HA-
300 ORP5. The advantage of analyzing ORP5 localization is its preferential localization to

301 contact sites as compared to ORP8 (Galmes et al. 2016), which remains also largely
302 present within the reticular ER. Interestingly, the majority of ORP5 gold particles was
303 found to localize to ER elements in a very close proximity (86% within 0-100 nm
304 distances, 50% of which within 50 nm) to the CJ (arrow, Fig. 2a-b), tubular structures
305 that connect the IMM to the cristae. In yeast, CJ were shown to be closely associated
306 to OMM-IMM contact sites tethered by the MICOS complex (Harner et al., 2011). IEM
307 analysis using Mitofilin-EGFP, an EGFP-tagged construct of the human orthologue of
308 the central component of the MICOS complex, confirmed that human Mitofilin,
309 similarly to its yeast orthologue, preferentially localizes to the IMM in close proximity
310 of CJ and in the cristae that arise from them (arrow, Fig. 2a).

311 To identify specific binding partners of ORP5 at ER-mitochondria contact sites
312 we carried out a MS-analysis on GFP-pull downs from cells expressing EGFP-ORP5,
313 EGFP-ORP5ΔPH (an ORP5 mutant lacking the PM-targeting PH domain that is
314 localized at ER-mitochondria but not at ER-PM contact sites), or EGFP alone as a
315 control (Fig. S2a). As expected, the highest hit detected in both EGFP-ORP5 and
316 EGFP-ORP5ΔPH pull-downs was its binding partner ORP8, with which ORP5 forms
317 a complex at contact sites (Chung, Torta et al., 2015, Galmes et al., 2016). Other
318 major hits are listed in Fig. S2a and comprise several outer mitochondrial membrane
319 proteins, including the mitochondrial outer membrane protein PTPIP51, a previously
320 reported interacting partner of ORP5 (Galmes et al., 2016).

321 Interestingly, among these proteins the Sorting Assembly Machinery Subunit 50
322 (SAM50), a central component of the SAM protein complex involved in the import
323 and assembly of mitochondria β-barrel proteins in the OMM (Hohr et al., 2018), had
324 one of the highest scores. SAM50 is part of the Mitochondrial intermembrane space
325 bridging (MIB) complex, composed of metaxin-1, metaxin-2 and the MICOS complex

326 that anchors the OMM to the IMM at CJ (Huynen et al., 2016, Ott et al., 2015).
327 SAM50 is also known to directly bind the central component of the MICOS complex
328 Mitofilin (Ott et al., 2015). Consistently, Mitofilin was also detected in the MS of
329 EGFP-ORP5 and EGFP-ORP5ΔPH pull-downs (Fig. S2a). Interestingly, SAM50 and
330 Mitofilin showed a higher interaction score in EGFP-ORP5ΔPH immunoprecipitates,
331 as compared to EGFP-ORP5. Of note, metaxin-2, another component of the MIB
332 complex, localized to the outer mitochondrial membrane, was also detected in the
333 MS of immunoprecipitated EGFP-ORP5ΔPH, whereas its score in the EGFP-ORP5
334 was lower than the assigned threshold (50) (Fig. S2a). To verify that the
335 overexpression of ORP5 or ORP5ΔPH did not affect the protein levels of SAM50 and
336 Mitofilin, WB analysis using anti-actin as loading control was carried out on cells
337 expressing EGFP-ORP5, EGFP-ORP5ΔPH or EGFP alone. Our results show that
338 neither EGFP-ORP5 nor EGFP-ORP5ΔPH overexpression alters the amount of
339 SAM50 and Mitofilin proteins as compared to the overexpression of EGFP alone (Fig.
340 S2b).

341 To confirm ORP5/8 interaction with SAM50 and Mitofilin, GFP-pull down
342 experiments from HeLa cells expressing EGFP-ORP5, EGFP-ORP8 or EGFP alone
343 were carried out (Fig. 2d). Consistent with the MS data, endogenous SAM50 and
344 Mitofilin were recovered with both EGFP-ORP5 and EGFP-ORP8 but not with EGFP
345 alone, confirming specific interaction of ORP5 and ORP8 with SAM50 and Mitofilin.
346 Of interest, the interaction of SAM50 and Mitofilin with EGFP-ORP5 was stronger
347 than with EGFP-ORP8.

348 Parallel GFP-pull down experiments were carried out from cells expressing
349 EGFP-tagged ORP5 (EGFP-ORP5ΔPH) or ORP8 (EGFP-ORP8ΔPH) PH domain
350 deleted constructs, and compared to the full-length proteins (EGFP-ORP5 and

351 EGFP-ORP8) or to the EGFP alone. In accord with the MS data, the deletion of the
352 PH domain increased ORP5 interaction with SAM50, as compared to the full-length
353 protein (Fig. 2e). In addition, EGFP-ORP8ΔPH interaction with SAM50 was higher
354 than of EGFP-ORP8, although still lower than the interaction of EGFP-ORP5ΔPH or
355 even EGFP-ORP5 with SAM50.

356 As the PH domain is not required for the interaction with SAM50 we further
357 investigated the role of the other domains of ORP5 in such interaction by
358 immunoprecipitating ORP5 deletion mutants for the ORD and the transmembrane
359 (TM) domains (EGFP-ORP5ΔORD, EGFP-ORP5ΔTM). While the deletion of the
360 ORD domain did not affect the interaction between ORP5 and SAM50, the deletion of
361 the TM domain decreased the amount of SAM50 co-immunoprecipitated with ORP5,
362 indicating that ORP5 should be properly anchored to the ER to localize at ER-
363 mitochondria contact sites and to interact with SAM50 (Fig. 2d).

364 To observe ORP5 and SAM50 localization we performed confocal analysis on
365 cells expressing EGFP-ORP5 or EGFP-ORP5ΔPH and stained with an anti-SAM50
366 antibody to look at the endogenous protein (Fig. 2e). As expected, endogenous
367 SAM50 localizes on mitochondria in cells stained with Mitotracker and, as previously
368 shown (Galmes et al., 2016), EGFP-ORP5 localizes to both ER-PM and ER-
369 mitochondria contact sites while the EGFP-ORP5ΔPH loses its localization to ER-PM
370 contacts. Both full-length and ΔPH constructs co-localized with endogenous SAM50
371 at ER elements in contact with mitochondria (Fig. 2e). However, this was more
372 evident in the case of EGFP-ORP5ΔPH (bottom image, Fig. 2e).

373 To test whether the interaction of ORP5 with the MIB complex could facilitate
374 the non-vesicular transfer of PS from the ER to the mitochondrial membranes we
375 depleted ORP5, SAM50 or Mitofilin alone or in combination by RNAi and analyzed

376 the content in mitochondrial PE newly synthesized from PS using the same
377 radiometric PS-to-PE conversion assay *in situ* described above. Robust
378 knockdown of ORP5, SAM50 or Mitofilin was confirmed by western blotting after
379 48 hours (Fig. 3a). Analysis of PS-derived newly synthetized PE revealed a
380 significant decrease in PE in ORP5 (Fig. 1d and Fig. 3b) and Mitofilin knockdown
381 cells (Fig. 3b). Moreover, the double-knockdown of ORP5 and Mitofilin had an
382 additive effect, supporting a cooperation of these two proteins in the same
383 process (PS transport/PE synthesis). However, the levels of PE were not changed
384 in SAM50 knockdown cells as compared to control. This can be explained by the
385 fact that other subunits of the MIB complex (i.e. Mitofilin) might compensate for its
386 depletion. Indeed, levels of Mitofilin are increased in SAM50 knockdown cells (Fig.
387 3a). Accordingly, the double-silencing of SAM50 and either ORP5 or Mitofilin had
388 a significant impact on PE synthesis (Fig. 3b). Moreover, the reduction in PE was
389 even stronger as compared to the individual knockdowns indicating that disruption
390 of both a component implicated in a direct transport of PS at ER-mitochondria
391 contact sites and a component of the OMM-IMM tethering complex has a
392 significant impact on PE synthesis.

393 To verify the possibility of an indirect effect of Mitofilin or SAM50 silencing on the
394 morphology and abundance of ER-mitochondria contacts we carried out a
395 morphological analysis by conventional EM in all these knockdown conditions and
396 quantified ER-mitochondria contacts by HRP-KDEL EM (to stain the ER) in Mitofilin
397 or SAM50 silenced cells. Morphological analysis by conventional EM showed that
398 transient (48 hours) knockdowns of SAM50 and Mitofilin, as well as double-
399 knockdowns of ORP5+Mitofilin, ORP5+SAM50 and SAM50+Mitofilin, induce
400 formation of multilamellar cristae, almost devoid of CJ (Fig. S3a-b), complementing

401 previous observations obtained by other groups through stable disruption of the
402 MICOS/MIB functions (Ding, Wu et al., 2015, Ott et al., 2015). However, in all
403 knockdown conditions analyzed ER-mitochondria contact sites were still present and
404 their morphology not altered. Quantitative morphological analysis by HRP-KDEL EM
405 in control and Mitofilin or SAM50 silenced cells confirmed that the abundance of ER-
406 mitochondria contact sites was not altered by Mitofilin or SAM50 knockdown (Fig. 3c-
407 d) indicating that the effects on PS-derived PE synthesis were specifically due to
408 Mitofilin or SAM50 loss of function effects on intra-mitochondrial membrane bridges.
409 Overall our results indicate that ER-mitochondria contacts where ORP5/8 localize
410 are physically linked to intra-mitochondria contacts where the components of the
411 MIB/MICOS complex reside, and this association facilitates the transfer of PS from
412 the OMM to the IMM for synthesis of PE.
413
414 **ORP5 additionally regulates VDAC-mediated Ca^{2+} import to mitochondria by**
415 **acting on the MIB component SAM50**
416 To get further insights into the functional link between ORP5/8 and the
417 MIB/MICOS complex we explored whether ORP5 and ORP8 knockdowns might
418 affect the expression and/or the localization of SAM50 and Mitofilin. Interestingly
419 when we analyzed cell lysates by WB, we found that SAM50 and Mitofilin protein
420 expression was significantly and specifically increased in ORP5- but not in ORP8
421 knockdown cells, as compared to control cells (Fig. 4a-b). The effects of ORP5
422 silencing were specific for SAM50 and Mitofilin, as other mitochondrial proteins
423 (Cytochrome c, CV-ATP5A) or proteins of the general ER (PDI) and of the MAMs
424 (VAPB) were not affected (Fig. S2c). This could also be observed by IF analysis
425 where the increase in SAM50 staining is specific to ORP5 knockdown (Fig. 4c).

426 Intriguingly, such increase in SAM50 was accompanied by a dramatic
427 accumulation in the voltage-dependent anion channel VDAC, a 19-stranded
428 transmembrane β-barrel protein (Colombini, 2012) that requires SAM50 for its
429 biogenesis and insertion in the OMM (Hohr et al., 2018). These results suggest that the
430 accumulation of VDAC protein in ORP5 knockdown cells is due to the parallel
431 increase of SAM50. The simultaneous knockdowns of ORP5 and SAM50 rescued
432 the increase in VDAC, confirming that its accumulation in ORP5 knockdown cells
433 was due to the accumulation of SAM50. Interestingly, ORP5 and SAM50 double-
434 knockdown did not rescue the increased protein levels of Mitofilin (Fig. 4a-b), in
435 accord with the fact that SAM50 knockdown alone also increased the protein levels
436 of Mitofilin. To verify that the increase in their protein amounts did not depend on the
437 increase in their transcripts we quantified the transcript levels of ORP5, SAM50 and
438 the three VDAC isoforms (VDAC1, 2 and 3) by real-time PCR upon ORP5 or SAM50
439 knockdown (Fig. 4d). The quantity of SAM50 and Mitofilin transcripts was unaffected
440 by ORP5 silencing, and VDAC1 transcript levels were even slightly reduced, possibly
441 reflecting a compensatory mechanism induced by the increase in VDAC protein.
442 Similarly, ORP5, VDAC and Mitofilin transcript levels were not affected in the
443 absence of SAM50. Overall, these data indicate that ORP5 knockdown induces an
444 accumulation of SAM50 and VDAC (SAM50-dependent) proteins in the absence of
445 major effects on the corresponding mRNAs.

446 As VDAC is one of the major mitochondrial players in mediating Ca^{2+} transport
447 and signaling between ER and mitochondria (Rapizzi, Pinton et al., 2002), we next
448 investigated if ORP5 knockdown could regulate Ca^{2+} influx from the ER to
449 mitochondria in living cells. We first used a ratiometric Ca^{2+} indicator selectively
450 targeted to mitochondria (mt-Pericam) which is based on a circularly permuted yellow

451 fluorescent protein fused to calmodulin (Nagai, Sawano et al., 2001). Upon binding to
452 Ca^{2+}, the excitation peak of mt-Pericam shifts from 415 nm to 494 nm while the
453 emission spectrum remains unchanged at 515 nm, allowing us to quantify rapid
454 changes in mitochondrial Ca^{2+}. We thus transfected ORP5, ORP8 or Ctrl siRNA-
455 treated cells with mt-Pericam and then we measured mitochondrial Ca^{2+} fluxes, in
456 response to histamine, by live-cell spinning disk microscopy (Fig. 5a-b). Soon after
457 the addition of histamine mt-Pericam fluorescence ratio (488/405) rapidly increased
458 in all conditions, reflecting the increase in mitochondrial Ca^{2+} influx triggered by the
459 histamine treatment. However, interestingly, ORP5 knockdown cells showed a
460 significantly higher increase as compared to ORP8 knockdown and to control cells.
461 Quantification of the maximum amplitude of the response after histamine treatment
462 (expressed as R_{max}/R_0, where R is the 488/405 ratio, R_{max} is the maximum
463 fluorescence ratio after histamine addition and R_0 the basal ratio before histamine
464 addition) confirmed that ORP5 but not ORP8 silencing induced a statistically relevant
465 increase of mitochondrial Ca^{2+} influx, triggered by the addition of histamine, as
466 compared to control cells (Fig. 5b).

467 Passive entry of Ca^{2+} into mitochondria can be driven by large negative
468 membrane potential of mitochondria and occurs through the mitochondrial Ca^{2+}
469 uniporter MCU spanning the IMM (Patron, Raffaello et al., 2013). To be sure that the
470 increase of mitochondrial Ca^{2+} upon ORP5 knockdown was not indirectly due to a
471 change of mitochondrial membrane potential, we performed flow cytometric analysis
472 in ORP5 or ORP8 knockdown cells. Knockdown cells were loaded with the
473 mitochondrial membrane potential sensitive dye TMRE (Tetramethylrhodamine ethyl
474 ester), in the presence (or absence) of the mitochondrial oxidative phosphorylation
475 uncoupler CCCP (Carbonyl cyanide 3-chlorophenylhydrazone) to induce membrane

476 depolarization (Fig. S4a). Neither ORP5 nor ORP8 knockdown altered mitochondrial
477 membrane potential as compared to control cells, even in presence of CCCP that
478 strongly decreases TMRE fluorescence intensity (Fig. S4a), showing that the effect of
479 ORP5 knockdown on Ca^{2+} is not due to a change of mitochondrial membrane
480 potential.

481 To further study the role of ORP5 in the regulation of Ca^{2+} homeostasis a parallel
482 set of experiments was performed using another type of Ca^{2+}-probe, the red
483 fluorescent genetically encoded Ca^{2+} indicator (R-GECO) and its two variants
484 selectively-targeted to mitochondria (mito-LAR-GECO) or ER (ER-LAR-GECO) to
485 respectively measure cytosolic, mitochondrial and ER Ca^{2+} in living cells (Fig. 5c-e).
486 These experiments confirmed that ORP5 silencing increases mitochondrial Ca^{2+}
487 uptake (Fig. 5c). The increase in mitochondrial Ca^{2+} uptake triggered by histamine
488 treatment was accompanied by a decrease in ER Ca^{2+} and an increase in cytosolic
489 Ca^{2+} in all conditions (Fig. 5d-e). Interestingly, no statistically relevant differences of
490 the decrease ER Ca^{2+} were measured between ORP5 silenced and control cells.
491 This indicates that the effects on mitochondrial Ca^{2+} fluxes observed in ORP5 do not
492 depend on the modification of ER Ca^{2+} storage or release. An increase in cytosolic Ca^{2+}
493 was measured in ORP5 and ORP8 silenced cells as compared to control. As this
494 increase was not specific of ORP5 silencing, it likely reflects a common role of
495 ORP5/8 in Ca^{2+} homeostasis at ER-PM contact sites, as recently proposed (Pulli,
496 Lassila et al., 2018).

497 So far, our data show that absence of ORP5 increases mitochondrial Ca^{2+}
498 possibly acting on the mitochondrial proteins SAM50 and VDAC and this is supported
499 by their specific accumulation in the absence of ORP5. To confirm that the increase
500 of mitochondrial Ca^{2+} in ORP5-silenced cells was indeed dependent on VDAC and

501 SAM50, we measured mitochondrial Ca^{2+} using mt-Pericam in VDAC- (all three
502 isoforms), ORP5+VDAC- and ORP5+SAM50- doubly-silenced cells. A significant
503 decrease in VDAC and SAM50 as well as ORP5 levels was detected by WB in the
504 corresponding silenced cells (Fig. 6a and Fig. 4a). Even though the increased level of
505 VDAC protein induced by the absence of ORP5 was not completely rescued in
506 ORP5+VDAC or ORP5+SAM50 knockdowns, the increase in mitochondrial Ca^{2+}
507 phenotype was significantly rescued to control levels and partially rescued in
508 ORP5+VDAC and ORP5+SAM50-silenced cells respectively (Fig. 6b and c). Overall,
509 these results demonstrated that ORP5 plays an additional role in regulating import of
510 mitochondrial Ca^{2+} through regulation of SAM50 and VDAC proteins.
511

512 **ORP5 knockdown induces cell senescence in a SAM50-dependent manner**

513 Accumulation of mitochondrial Ca^{2+} has been shown to be involved in both
514 apoptotic cell death and senescence (permanent cell proliferation arrest) (Wiel,
515 Lallet-Daher et al., 2014). Interestingly, it also has been shown that ORP5 expression
516 promotes cell proliferation (Du, Zadoorian et al., 2018). As our data show a new role
517 of ORP5 in regulating Ca^{2+} import to mitochondria at ER-mitochondria contact sites
518 (Fig. 6), we were wondering if ORP5 could mediate cellular processes such as
519 apoptosis and senescence. To address this question, we decided to study both
520 apoptosis and senescence in ORP5 depleted cells.

521 For apoptosis, we performed immunofluorescence and stained with anti-cleaved
522 caspase-3 (to detect apoptotic cells) HeLa cells transfected with control siRNA or
523 siRNA against ORP5 or ORP8. We also treated the knockdown cells with
524 staurosporine to induce apoptosis or with DMSO as control (Fig. S4b). Quantification
525 of the percentage of cleaved caspase-3 positive cells (% of total cells) in all

526 conditions showed no significant difference between ORPs knockdown and control
527 cells (Fig. S4b), even in presence of staurosporine, showing that in these conditions
528 (transient silencing) ORP5/8 are not mediating apoptosis.

529 To study cellular senescence we performed knockdown of ORP5 in MRC5
530 normal human fibroblasts that are a good model for senescence studies (Ma,
531 Warnier et al., 2018). We first looked at cell proliferation using crystal violet staining
532 in both control and ORP5 knockdown cells (Fig. 7a). A significant decrease of the cell
533 number was detected in ORP5 depleted cells compared to control (Fig. 7a). This
534 decrease of the cell number in ORP5 knockdown cells could be correlated with an
535 increase of the Senescence-associated β –galactosidase (SA β-gal) staining in this
536 condition as compared to control (Fig. 7a). Accordingly, these results were
537 corroborated by qRT-PCR analysis showing a significant increase of senescence
538 markers p16 and p21 and a significant decrease of the proliferation marker Ki67 in
539 ORP5 knockdown cells compared to control (Fig. 7b). Because we have shown that
540 ORP5 knockdown induces an elevation of mitochondrial Ca^{2+} via a SAM50-
541 dependent accumulation of VDAC, we hypothesized that the induction of senescence
542 in MRC5 cells was also due to the effect of ORP5 on SAM50. Thus, we decided to
543 perform a double-knockdown of ORP5 and SAM50 in MCR5 cells. In accord with our
544 hypothesis, double-silencing of ORP5 and SAM50 partially rescued the senescence
545 phenotype found in ORP5 knockdown cells. ORP5+SAM50 double-knockdown cells
546 displayed increased proliferation and underwent less senescence as compared to
547 ORP5 single knockdown cells (Fig. 7c). To verify that this effect on cellular
548 senescence was specific to ORP5, given its role on mitochondria Ca^{2+}, we performed
549 in parallel a knockdown of ORP8 in MRC5 cells. Differently to ORP5, ORP8
550 knockdown did not induce cell senescence (Fig. 7c). Taken together, these data

551 reveal a novel specific role of ORP5 and involving SAM50 in regulating cellular
552 senescence in MRC5 cells.
553

554 **DISCUSSION**

555 In this study, by using a combination of *in vitro* and *in situ* biochemical and imaging
556 approaches, we describe a new function of ORP5/8 in the maintenance of mitochondrial
557 levels of PE, an essential phospholipid of mitochondria, in mammalian cells. Importantly,
558 we provide the first evidence of LTPs (ORP5 and ORP8) directly mediating non-
559 vesicular transfer of PS at ER-mitochondria contact sites, from the ER to the
560 mitochondria where it is converted into PE.

561 In yeast, large complexes such as the ERMES and the ER-Membrane protein
562 Complex (EMC) have been previously involved in phospholipid trafficking (PC and
563 PS, respectively) between the ER and the mitochondrial membranes (Tamura &
564 Endo, 2017) (Lang, John Peter et al., 2015). For instance, three subunits of the
565 ERMES complex contain a Synaptotagmin-like Mitochondria lipid binding Protein
566 (SMP) domain that forms a hydrophobic lipid transport cavity shared by other
567 proteins of the tubular lipid-binding protein TULIP superfamily (Kopec, Alva et al.,
568 2010, Reinisch & De Camilli, 2016). However, a direct role (independent of its
569 tethering function) of ERMES in lipid transport at ER-mitochondria contact sites is still
570 questioned. In addition, the ERMES complex is not conserved in metazoans. The
571 EMC complex, which has been involved in PS shuttling at ER-mitochondria contact
572 sites in yeast, is instead highly conserved in metazoans (Wideman, 2015). However,
573 no lipid-binding/transfer domain has been found in the EMC proteins, indicating that
574 their implication in lipid transfer at ER-mitochondria contacts is linked to their
575 tethering function rather than to a direct lipid transfer activity. Recently, the

576 mammalian LTP VPS13A has been shown to localize to contact sites including ER-
577 mitochondria contacts. VPS13A contains a lipid-binding domain (VPS13α) that has
578 the ability to harbor multiple phospholipids at once and transfer them between
579 liposomes *in vitro*. However its role in lipid transfer at membrane contact sites *in situ*
580 has not been established yet. Differently from the SMP and VPS13α that can
581 simultaneously host multiple phospholipids, the ORD domain of Osh/ORPs forms a
582 cavity that can host only one lipid at a time (Maeda, Anand et al., 2013, Wang, Ma et
583 al., 2019).

584 ORP5 and ORP8 have recently been shown to counter-exchange PS with PM
585 phosphoinositides PI4P and PIP_2 at ER-PM contact sites in HeLa cells (Chung et al.,
586 2015, Ghai, Du et al., 2017). However PI4P and PIP_2 are not present on the
587 mitochondrial membranes, while PE is highly abundant on these membranes, in
588 addition to being an essential lipid of all biological membranes. Our *in vitro* data show
589 that the ORD domains of ORP5 and ORP8 transport PS, but not other phospholipids
590 such as PE and PC, indicating a specific role of ORP5/8 in PS transport and excluding
591 the possibility that ORP5/8 might also participate in the transport of a fraction of PE
592 back to the ER. It is possible that ORP5/8 cooperate with other LTPs, such as VPS13A,
593 for the exchange of other lipids (including PE) at ER-mitochondria contact sites.
594 Importantly, we have confirmed the role of ORP5/8 in PS transfer by measuring a
595 decrease of PS-derived mitochondrial PE in ORP5 (and even more in ORP5+8)
596 depleted HeLa cells *in situ*. Accordingly, the knockdown of ORP5/8 affects cristae
597 morphology (Fig.1e) and the respiratory function of mitochondria (Galmes et al.,
598 2016), all phenotypes that are expected in the case of decrease in mitochondrial PE
599 (Joshi et al., 2012, Steenbergen et al., 2005). Our data further confirm the essential
600 role of PE in the maintenance of mitochondria structure and functions, and are in

601 accord with those of (Tasseva et al., 2013) showing that even a mild decrease of
602 mitochondrial PE can strongly alter mitochondria cristae morphology and respiratory
603 function. Our data also suggest that the gradient of PS at ER-mitochondria contacts
604 is sufficient to trigger the ORP5/8-mediated unidirectional transport of PS from the
605 MAMs, where it is highly enriched, to the mitochondria membranes, where it is
606 rapidly converted into PE and is therefore present at a very low amount. Our findings
607 have important implications in the general field of LTPs, as they suggest that the same
608 LTP can use different means to transfer lipids depending on the local gradients
609 present at the specific membrane contact sites where it is localized. They also
610 suggest a possible new function in PS transport at ER-mitochondria contact sites for the
611 yeast orthologues of ORP5/8, Osh6/7p, whose localization/function at ER-mitochondria
612 contact sites still remain to be addressed (Moser von Filseck, Copic et al., 2015).

613 Another important feature of our work is the observation that ORP5 and ORP8
614 knockdowns do not affect the extent of ER-mitochondria contact sites. These data,
615 consistent with our previous observation that ORP5/8 overexpression does not alter
616 the length of ER-mitochondria contacts (Galmes et al., 2016), confirm that the main
617 function of these proteins at MAMs is lipid transfer and not membrane tethering. This
618 is a unique feature among the LTPs that have been identified so far at MAMs. For
619 instance, modulation of the protein levels of other LTPs such as ERMES in yeast, or
620 VPS13A in mammals, results in an alteration of the extent of ER-mitochondria
621 contact sites (Kornmann, Currie et al., 2009, Kumar, Leonzino et al., 2018). Thus,
622 ORP5/8 represent so far a unique tool to study lipid transport at ER-mitochondria
623 contact sites, independently of membrane tethering.

624 Importantly, our study also reveals that ORP5/8 physically interact with SAM50
625 and Mitofilin, two key components of the MIB/MICOS complex that anchor the IMM to

626 the OMM at the level of CJ (Huynen et al., 2016, Ott et al., 2015, Wollweber et al.,
627 2017). The biochemical interaction between ORP5 and SAM50/Mitofilin suggests the
628 existence of a physical link between ER-mitochondria contact sites involved in lipid
629 transport and intra-mitochondria contacts. ORP5 localization by IEM at ER-mitochondria
630 contact sites near the CJ, where Mitofilin and MICOS complex also reside, confirms the
631 existence of such tripartite membrane structure. Importantly, we also show that ORP5
632 cooperates with SAM50 and Mitofilin to facilitate the movement of PS from the ER to
633 the IMM across ER-mitochondria contact sites and the IMS. Taken together these
634 findings provide the first evidence of a physical link between ER-OMM and OMM-
635 IMM contact sites to facilitate transport of PS from the ER to the mitochondria.

636 Finally, we uncover a specific role for ORP5, but not ORP8, in regulating
637 mitochondrial Ca^{2+} uptake, via a SAM50-VDAC axis. SAM50, in addition to be part of
638 the MIB complex, is also the major subunit of the Sorting and Assembly Machinery
639 (SAM) that mediates membrane insertion and assembly of mitochondrial β-barrel
640 proteins (Hohr et al., 2018, Kozjak et al., 2003). One of the most abundant
641 mitochondria β-barrel proteins is VDAC, which forms a pore that allows the passage of
642 small metabolites and ions such as ATP and Ca^{2+} across the OMM (Colombini, 2012).
643 VDAC exists as three isoforms (VDAC1, 2 and 3) that share common channeling
644 properties. VDAC interacts with the IP3Rs located in the ER membrane via the
645 molecular chaperone GRP75 that links the two proteins together. The interaction
646 between IP3Rs and VDAC acts as a bridge to allow the transfer of Ca^{2+} from the ER to
647 the OMM, and then from the OMM to the mitochondrial matrix through the mitochondrial
648 Ca^{2+} uniporter MCU. Ca^{2+} release from the ER to mitochondria is triggered by IP_3
649 binding to IP3Rs upon histamine treatment (Rizzuto, Marchi et al., 2009). Here we show
650 that knockdown of ORP5 increases the protein levels of SAM50 and VDAC as well as

651 the levels of histamine-induced Ca^{2+} import to mitochondria. The additional function of
652 ORP5 in regulating mitochondrial Ca^{2+} uptake is most likely independent on its direct
653 function in mediating PS transport, carried out in complex with ORP8, and this is
654 consistent with the lack of effect of ORP8 knockdown on SAM50-VDAC and
655 mitochondrial Ca^{2+} levels. Importantly, double-knockdown of ORP5 and VDAC rescues
656 the mitochondrial Ca^{2+} increase induced by ORP5 silencing, confirming that this increase
657 in Ca^{2+} is indeed VDAC-dependent. Mitochondrial membrane potential has been
658 proposed to influence VDAC open-closed state and consequently import of Ca^{2+} to
659 mitochondria (Tan & Colombini, 2007). However, we did not detect any alteration in
660 mitochondria membrane potential in ORP5 knockdown cells, supporting our idea that
661 the increase in mitochondrial Ca^{2+} observed in ORP5-silenced cells is related to the
662 increase in VDAC protein levels. This is also in accord with previous studies showing
663 that the overexpression of VDAC in HeLa cells enhances mitochondrial Ca^{2+} uptake
664 upon histamine treatment (Rapizzi et al., 2002). Our data also indicate that the effect of
665 VDAC accumulation on mitochondrial Ca^{2+} fluxes does not depend on the modification
666 of ER Ca^{2+} storage or release. Indeed, we observe a similar decrease of ER Ca^{2+} in
667 ORP5 depleted and control cells, upon histamine treatment. Our results correlate well
668 with those of others (Rapizzi et al., 2002) who also observed similar decrease of ER
669 Ca^{2+} in VDAC-overexpressing and control cells.

670 Accumulation of Ca^{2+} in mitochondria, induced by release of Ca^{2+} through IP3R
671 channel, has been recently shown to be involved in cellular senescence in normal
672 cells (Martin & Bernard, 2018, Wiel et al., 2014). Interestingly, we found that
673 knockdown of ORP5, but not ORP8, induces cellular senescence in MCR5 cells.
674 These findings are highly relevant because ORP5 overexpression has been
675 associated to several tumors (Du, Turner et al., 2017). We also show that double-

knockdown of ORP5 and SAM50 restores the levels of VDAC protein, as well as of mitochondrial Ca^{2+}, and enables cells to escape from the senescence fate induced by ORP5 depletion. These data confirm that all these phenotypes are dependent on SAM50, and possibly on its known role in the biogenesis of VDAC (Hohr et al., 2018). Future studies are required to further dissect the mechanisms underlying the role of ORP5 in modulating SAM50 protein levels in mitochondria.

In conclusion, our data reveal that: 1) ORP5/8 constitute the molecular machinery mediating PS transfer at ER-mitochondria contact sites; 2) ER-mitochondria contacts where ORP5/8 localize are physically associated with intra-mitochondrial contacts to facilitate the transport of PS from the ER to mitochondria membranes; 3) ORP5 plays a novel specific role in regulating cellular senescence in MCR5 cells by modulating mitochondrial Ca^{2+} fluxes at ER-mitochondria contact sites via SAM50/VDAC (Fig. 8). Our study uncovers a novel link between lipid and Ca^{2+} transport at ER-mitochondria contact sites and provides a first molecular clue on how lipids are transported at ER-mitochondria contact sites. Furthermore, we reveal a specific role of ORP5 in regulating mitochondrial Ca^{2+} homeostasis and cellular senescence in mammalian cells.

701 **METHODS**

702 **Cell culture, siRNA and transfection**.

703 HeLa cells were cultured in DMEM (Life Technologies) containing GlutaMax (Life
704 Technologies) and supplemented with 10% FBS (Life Technologies) and 1%
705 penicillin/streptomycin (Life Technologies) at 37°C and 5% CO_2. Transfection of
706 plasmids and siRNA oligos (Dharmacon, GE Healthcare) was carried out with
707 lipofectamine 2000 and oligofectamine (Life Technologies) according to
708 manufacturer's instructions.

709 For senescence studies, MRC5 normal human fibroblasts (ATCC) were cultured in
710 Dulbecco′s modified Eagle′s medium (Life Technologies) containing GlutaMax (Life
711 Technologies) and supplemented with 10% FBS (Sigma-Aldrich) and 1%
712 penicillin/streptomycin (Life Technologies). MRC5 were transfected with siRNAs
713 using Dharmafect 1 transfection reagent according to manufacturer's instructions
714 (Dharmacon, GE Healthcare).

715

716 **siRNAs oligonucleotides**

717 For knockdowns, HeLa cells were transfected with siRNA oligos by using
718 oligofectamine (Life Technologies) and cultured for 48 hours prior to analysis.

719 For senescence studies, MRC5 cells were transfected with siRNAs using Dharmafect
720 1 transfection reagent according to manufacturer's instructions (Dharmacon, GE
721 Healthcare).

722 Double-stranded siRNAs were derived from the following references:

siRNA	Company, Reference
OSBPL8	Dharmacon, J-009508-06 (Galmes et al., 2016)
OSBPL8	Dharmacon, J-009508-05 (Galmes et al., 2016)

OSBPL5	Dharmacon, J-009274-10 (Galmes et al., 2016)
OSBPL5	Dharmacon, J-009274-11 (Galmes et al., 2016)
SAMM50	Dharmacon, J-017871-18
SAMM50	Dharmacon, J-017871-19
Non-targeting	Dharmacon, D-001810-10
hVDAC1	5'-AAGCGGGAGCACATTAACCTG-3' (De Stefani, Bononi et al., 2012)
hVDAC2	5'-AAGGATGATCTCAACAAGAGC-3' (De Stefani et al., 2012)
VDAC3	5'-AAGGGTGGCTTGCTGGCTATC-3' (De Stefani et al., 2012)
siMitofilin	5' -AAUUGCUGGAGCUGGCCUUTT-3' (John, Shang et al., 2005)

723

724 **Plasmids and cDNA clones**

725 EGFP-ORP5, EGFP-ORP8, EGFP-ORP5ΔPH, EGFP-ORP8ΔPH, EGFP-
726 ORP5ΔORD and EGFP-ORP5ΔTM were described in (Galmes et al., 2016). The
727 following reagents were kind gifts: 2mt RP ratiometric Pericam from Paolo Pinton
728 (University of Ferrara) (Filippin, Abad et al., 2005); ssHRP-KDEL from T. Schikorski
729 (Schikorski, Young et al., 2007); GST-ORD8 (ORD ORP8, corresponding to aa 328-
730 767) from P. De Camilli (Chung et al., 2015). GECO constructs were from Robert
731 Campbell (Addgene: CMV-R-GECO1 # 32444, CMV-mito-R-GECO1 # 46021, CMV-
732 ER-LAR-GECO1 # 61244, CMV-mito-LAR-GECO1.2 # 61245).

733

734 **Cloning of HA-ORP5, Mitofilin-GFP and GST- ORD5 (ORD ORP5)**

735 cDNAs of ORP5 (full-length), Mitoflin (full-length from FLAG-mitofilin (Ott et al.,
736 2015)) and GST-ORD5 (corresponding to aa 265-703), were amplified by PCR. In all
737 PCR reactions, Herculase II fusion DNA polymerase (Agilent) was used. Primers
738 used were (coding sequence shown in lowercase):

739 5’ AgeI-HA-ORP5_Fw GGCGGC *ACCGGT* cgccacc ATGTACCCATACGATGTTCCA
740 GATTACGCT atgaaggaggaggccttcctc
741 3’ XhoI-STOP-ORP5_Rv GGC *CTCGAG* ctatttgaggatgtggttaatg
742 5’ KpnI-Mitofilin_Fw AGACCCAAGCTT *GGTACC* atg
743 3’ BamHI-GC-Mitofilin_Rv GTAATC *GGATTC* GC ctctggct
744 5’ SalI-TC-ORD5_Fw GCACAG GTCGAC TC gagacccctggggccccggt
745 3’ NotI-STOP-ORD5_Rv GCACA *GCGGCCGC* ctactgtggccggagggctggtcg
746 For the HA-ORP5 cloning the PCR product (carrying the HA tag at the N-terminus of
747 ORP5) was ligated between AgeI and XhoI in the pEGFP-C1 vector (Clontech) and
748 replacing the GFP- with the HA-tag. For the other clonings the PCR products were
749 ligated between KpnI and BamHI for Mitofilin, and between SalI and NotI for ORD5,
750 in the pEGFP-N1 vector (Clontech) to generate Mitofilin-EGFP or in the pGEX-6P-1
751 to generate GST-ORD5.
752
753 **mRNA analyses by quantitative by reverse transcriptase PCR (qPCR)**

754 Total RNA was isolated from HeLa cells transfected with siRNAs for 48 hours as
755 described above, by using a Purelink™ kit (Ambion/Thermo Scientific, Foster City,
756 CA). The RNA (0.5 μg per specimen) was reverse transcribed with a SuperScript
757 VILO™ cDNA synthesis kit (Invitrogen/Thermo Scientific, Carlsbad, CA) according to
758 the manufacturer’s protocol. Quantification of the mRNAs of interest was carried out
759 on a Roche Lightcycler™ 480 II instrument by using SYBR-Green I Master mix
760 (Roche, Basel, Switzerland) and primers specified in Table SX. Succinate
761 dehydrogenase complex. subunit A, the mRNA of which remained markedly stable
762 under the present conditions, was employed as a reference housekeeping mRNA.
763 Relative mRNA levels were calculated by using the –ΔΔCt method.

764 Sequences of the primers used for qPCR:

mRNA	Sequence 5'-3'
SDHA* (housekeeping)	Fw: CATGCTGCCGTGTTCCGTGTGGG
	Rv: GGACAGGGTGTGCTTCCTCCAGTGCTCC
ORP5	Fw: GTGCCGCTGGAGGAGCAGAC
	Rv: AGGGGCTGTGGTCCTCGTATC
SAMM50	Fw: CAAGTGGACCTGATTTTGGAGG
	Rv: AGACGGAGCAATTTTTCACGG
Mitofilin	Fw: GTTGTATCTCAGTATCATGAGCTGG
	Rv: GTTCAGCTGATCAATACGACGA
VDAC1	Fw: ACGTATGCCGATCTTGGCAAA
	Rv: TCAGGCCGTACTCAGTCCATC
VDAC2	Fw: GCTACAGGACTGGGGACTTC
	Rv: AATGCCAAAACGAGTGCAGTT
VDAC3	Fw: GTAAATAATGCCAGCCTGATTG
	Rv: CTTCCAGGGACAAATCTGATG

765 *succinate dehydrogenase complex, subunit A

766

767 For senescence studies, MCR5 cells RNAs were extracted using RNA Extracol lysis
768 buffer (Dutscher). cDNAs were synthesized from total RNA using the Maxima first
769 strand cDNA synthesis kit (Life Technologies). Quantitative PCR mixture contained
770 cDNA, TaqMan mix (Roche), 100 µM of a Universal Probe Library probe (Roche) and
771 200 nM of primers (Sigma-Aldrich) according to manufacturer's instructions. qPCRs
772 were carried out on a FX96 Thermocycler (Bio-Rad). qPCR reactions were as
773 follows: 95°C 10 min, followed with 40 cycles of 95°C 10s, 59°C 30s. The reactions
774 were performed at least in duplicate. The relative amount of mRNA (ORP5, p16, p21,
775 Ki67, PGK1 and HPRT1) was calculated using the comparative Ct (ΔΔCt) method
776 and data were normalized using two housekeeping genes (PGK1 and HPRT1).

777

778 Sequences of the primers and UPL probes used for qPCR:

mRNA/UPL probe	Sequence 5'-3'
CDKN2A (p16) / UPL 34	Fw: GTGGACCTGGCTGAGGAG
	Rv: CTTTCAATCGGGGATGTCTG
p21(CDKN1A) / UPL 32	Fw: TCACTGTCTTGTACCCTTGTGC
	Rv: GGCGTTTGGAGTGGTAGAAAT
Ki67 / UPL 32	Fw: TCAAGGAACTGATTCAGGAGAAG
	Rv: GTGCACTGAAGAACACATTTCC
ORP5 / UPL 17	Fw: GTTGTATCTCAGTATCATGAGCTGG
	Rv: GTTCAGCTGATCAATACGACGA
PGK1 / UPL probe 67	Fw: CAGCTGCTGGGTCTGTCAT
	Rv: GCTGGCTCGGCTTTAACC
HPRT1 / UPL probe 73	Fw: TGACCTTGATTTATTTTGCATACC
	Rv: CGAGCAAGACGTTCAGTCCT

779

780

781 **Antibodies, dyes and reagents list**

782 Primary antibodies used in this study were:

	Antibody	Company & Reference
WB	IP3R-3	BD Transduction Laboratories, 610312
WB	ORP5	SIGMA, HPA038712
WB	ORP5	SIGMA, HPA058727
WB	beta-Actin	Abcam, ab8226
WB	ORP8	GeneTex, GTX121273
IF, WB	SAMM50	SIGMA, HPA034537
WB	Mitofilin	Proteintech, 10179-1-AP
WB	VDAC1	Abcam, ab15895
WB	Cytochrome C	BD Pharmingen, 556433
WB	Total OXPHOS CV-ATP5A	Abcam, ab110413

WB	PDI	GeneTex, GTX30716
WB	VAPB	SIGMA, HPA013144
IF, WB	GFP	Roche, 11814460001
IEM	GFP	Life technologies, A11122
IF	HA	SIGMA, H3663
IF	Cleaved caspase-3	Cell Signaling, 9661
IF	FLAG M2	SIGMA, F1804
IF	MitoTracker® Red CMXRos	M7512, Invitrogen
IF	MitoTracker® Deep Red FM	M22426, Invitrogen

783

784 **Biochemical analyses**

785 ***Western blot***

786 For immunoblotting, cells were resuspended in lysis buffer [50 mM Tris, 150 mM
787 NaCl, 1% Triton X-100, 10 mM EDTA, pH 7.2, and protease inhibitor cocktail
788 (Roche)]. Cell lysates were then centrifuged at 21 000 g for 20 min at 4°C. The
789 supernatants were boiled in reducing SDS sample buffer and proteins were
790 separated using 10% SDS-PAGE and immunoblot was carried using standard
791 methods.

792 For Western blot quantification, bands of protein of interest were detected using
793 ChemiDoc™ Imaging Systems (Life Science Research, Bio-Rad) and analyzed using
794 Image Lab™ Software. All data are presented as mean ±SEM of three experimental
795 replicates.

796 ***Immunoprecipitation of ORPs***

797 HeLa cells transfected with EGFP-tagged ORPs were washed in cold PBS and lysed
798 on ice in lysis buffer [50 mM Tris, 120 mM NaCl, 40 mM Hepes, 0,5% digitonin, 0,5%

799 CHAPS, pH 7.36, and protease inhibitor cocktail (Roche). Cell lysates were then
800 centrifuged at 21 000 g for 20 min at 4°C. Supernatants were then incubated with
801 Chromotek GFP-trap agarose beads (Allele Biotech) for 1 hour at 4°C under rotation.
802 Subsequently beads were washed in 0.1 M phosphate buffer. After extensive washes
803 in cold lysis buffer, immunoprecipitated proteins bound to the beads were processed
804 for Mass Spectrometry analysis (see below) or incubated in sample buffer (containing
805 2% SDS) and then boiled for 1 min. In the latter case immunoprecipitates were
806 loaded and separated in 10% SDS–PAGE gel and immunoblotting was carried out.

807 ***Cell fractionation***

808 HeLa cells ($100x10^6$ cells) were harvested 48 hours after transfection with siRNA
809 oligos and washed with PBS by centrifugation at 600 g for 5 min. The cell pellet was
810 resuspended in starting buffer (225 mM mannitol, 75 mM sucrose and 30 mM Tris-
811 HCl pH 7.4) and homogenized using a Tissue Grinder Dura-Grind®, Stainless Steel,
812 Dounce (Wheaton). The homogenate was centrifuged three times at 600 g for 5 min
813 to remove nuclei and unbroken cells. The crude mitochondria was pelleted by
814 centrifugation at 10 000 g for 10 min. To separate MAM and pure mitochondria
815 fractions, the pellet was resuspended in MRB buffer (250 mM mannitol, 5 mM
816 HEPES and 0.5 mM EGTA, pH 7.4) and layered on top of different concentrations of
817 Percoll gradient (225 mM mannitol, 25 mM HEPES, 1 mM EGTA pH 7.4 and 30% or
818 15% Percoll). After centrifugation at 95 000 g for 30 min, two dense bands containing
819 either the pure mitochondria or MAM fraction were recovered and washed twice with
820 MRB buffer by centrifugation at 6300 g for 10 min to remove residual Percoll and
821 residual contamination. MAM was pelleted by centrifugation at 100 000 g for 1 hour.
822 MAMs and pure mitochondria pellets were resuspended in Lysis Buffer (50 mM Tris,
823 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, pH 7.2, and protease inhibitor

824 cocktail) and protein concentrations were determined by Bradford assay. Equal
825 amount of proteins were loaded on 4-20% gradient SDS-PAGE gels (Biorad) and
826 immunoblotting was carried out. Pure mitochondria were processed for MS-lipidomic
827 analysis (see below).

828 ***Mass spectrometry analysis***

829 Mass Spectrometry (MS) analysis was carried out by the proteomics/mass
830 spectrometry platform in IJM (http://www.ijm.fr/plateformes/spectrometrie-de-masse).
831 Briefly, after washes with binding buffer, immunoprecipitations beads were rinsed
832 with 100 µl of NH4HCO3 25 mmol/l. Proteins on beads were digested overnight at
833 37°C by sequencing grade trypsin (12,5 µg/ml; Promega Madison, Wi, USA) in 20 µl
834 of NH4HCO3 25 mmol/l. Digests were analysed by an Orbitrap Fusion (Thermo
835 Fisher Scientific, San Jose, CA) equipped with a Thermo Scientific EASY-Spray
836 nanoelectrospray ion source and coupled to an Easy nano-LC Proxeon 1000 system
837 (Thermo Fisher Scientific, San Jose, CA). MS/MS data were processed with
838 Proteome Discoverer 1.4 software (Thermo Fisher scientific, San Jose, CA) coupled
839 to an in house Mascot search server (Matrix Science, Boston, MA ; version 2.4.2).
840 MS/MS datas were searched against SwissProt databases with Homo sapiens
841 taxonomy.

842

843 **Mass Spectrometry-lipidomic analysis**

844 700 µl of homogenized cells were mixed with 800 µl 1 N HCl:CH_3OH 1:8 (v/v), 900 µl
845 $CHCl_3$ and 200 µg/ml of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT; Sigma
846 Aldrich). The organic fraction was evaporated using a Savant Speedvac spd111v
847 (Thermo Fisher Scientific). Lipid pellets were reconstituted in running solution
848 (CH_3OH:$CHCl_3$:NH_4OH; 90:10:1.25; v/v/v). Phospholipid species were analyzed by

849 electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid triple
850 quadrupole/linear ion trap mass spectrometer (4000 QTRAP system; Applied
851 Biosystems SCIEX) equipped with a TriVersa NanoMate (Advion Biosciences)
852 robotic nanosource. Phospholipid profiling was executed by (positive or negative)
853 precursor ion or neutral loss scanning at a collision energy of 35 eV for neutral loss
854 141 (phosphatidylethanolamine (PE)). Phospholipid quantification was performed by
855 multiple reaction monitoring (MRM), the transitions being based on the neutral losses
856 or the typical product ions as described above. The MRM dwell time was set to 100
857 ms and typically the signal was averaged over 20 cycles. Lipid standards used were
858 PE25:0 and PE43:6 (Avanti Polar Lipids). The data were corrected for isotope effects
859 as described by (Liebisch, Lieser et al., 2004).
860

861 **Lipid Transfer assay**

862 ***ORP5 and ORP8 ORD domain purification***

863 Escherichia coli BL21DE3 RILP (Invitrogen) cells were transformed with plasmids
864 encoding for GST tagged ORP5 or ORP8 ORD domains following the manufacturer's
865 instruction. Bacteria were then grown overnight at 37°C and used to inoculate a
866 large-scale volume (1L). When the OD_{600} reached 0.4, cultures were cool down and
867 incubate at 18°C until they reached O_{D600} = 0.65. Cells were induced by addition
868 of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.1 mM and were
869 incubated overnight at 18°C before harvesting. Cells were resuspended in 35 ml
870 binding buffer (1X PBS, 1 mM EDTA, 1 mM DTT, Protease inhibitor) then 250 units
871 of benzonase nuclease (Sigma) were added to the resuspension. Cells were lysed by
872 sonication and the supernatant was recover after 20 min centrifugation at 184 000g
873 and 4°C. Supernatant containing GST tagged proteins was incubated with 2 ml of

874 Glutathione Sepharose 4 fast flow for 1 hour at 4°C under nutation. Beads were
875 washed using a serie of wash buffer: 1st (1X PBS, 1 mM EDTA, 1 mM DTT), 2nd HSP-
876 removal buffer (50 mM Tris pH7.5, 50 mM KCl, 20 mM MgCl2, 5 mM ATP) then
877 cleavage buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT).
878 Cleavage of the GST tag was realized overnight at 4°C using Prescission protease.
879 Untagged proteins were eluted with cleavage buffer, flash frozen and stored at -80°C
880 until lipid transfer assay was performed.

881 ***Lipids***

882 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-
883 phosphoethanolamine-N-(cap biotinyl) (Biotinyl Cap PE), 1-palmitoyl-2-
884 (dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphoethanolamine
885 (TopFluor-PE), 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-
886 glycero-3-phospho-L-serine (TopFluor-PS), 1-palmitoyl-2-(dipyrrometheneboron
887 difluoride)undecanoyl-sn-glycero-3-phosphocholine (TopFluor-PC) were purchased
888 from Avanti Polar Lipids as chloroform solutions.

889 ***Liposome preparation***

890 1 µmol of the appropriate lipid mixtures in chloroform solution was dried in glass
891 tubes for 10 min under a gentle stream of argon, and then for 1 hour under vacuum.
892 The dried lipid films were resuspended in 1 ml of buffer H (25 mM HEPES/KOH, pH
893 7.7; 150 mM KCL; 10% Glycerol) by vigorously vortexing for 30 min at room
894 temperature. Unilamellar liposomes were produced by seven freeze-thaw cycles (30
895 sec in liquid nitrogen followed by 5 min in a 37°C water bath) and extrusion (at least
896 21 times) through a polycarbonate filter with 100 nm pore size (polycarbonate
897 membranes from Avanti Polar Lipids). The liposomes were then stored on ice.

898 ***Lipid Transfer assay in vitro***

899 The lipid transfer assays were realized with liposomes prepared as described above.
900 The donor liposomes contained 1% mol TopFluor lipids (-PS, -PC or -PE) and 2%
901 mol of Biotinyl Cap PE. The acceptor liposomes contained only POPC. For each
902 reaction, 25 µl of streptavidin-coated magnetic beads (DynabeadsMyOne
903 Streptavidin T1, Invitrogen) were washed in buffer H and mixed with 25 µl of 1 mM
904 donor liposomes. The mixture was incubated for 1 hour at 25°C with intermittent
905 gentle mixing. Bead-bound donor liposomes were then washed, resuspended in 25µl
906 and mixed with 25µl of 1 mM acceptor liposomes and 50µl of buffer H or protein (0.3
907 µM protein and 2.5 µM TopFluor lipids in the reaction solution). The mixture was
908 incubated at 37°C for 1 hour with intermittent gentle mixing. Supernatant containing
909 acceptor liposomes was recovered after binding of bead-bound donor liposomes to a
910 magnetic rack. TopFluor fluorescence of acceptor and donor liposomes was
911 measured (after solubilization with 0.4% (w/v) n-dodecyl-β-D-maltoside) in a
912 SpectraMax M5 plate reader (Molecular Device) equilibrated to 30°C (excitation: 450
913 nm; emission: 510 nm; cutoff: 475 nm; low gain). The percentage of lipids transferred
914 from donor to acceptor liposomes was determined using the following formula:
915 $100*F_{acceptor}/(F_{acceptor}+F_{donor})$.

916

917 **Radiometric assay for the conversion of PS to PE**

918 Hela cells were seeded on 6-well plates and transfected for 48 hours with the non-
919 targeting, ORP5 or ORP8-specific siRNAs specified above by using Oligofectamine
920 (Thermo Fisher Scientific). The cells were then washed and shifted into Hanks
921 balanced salt solution (Gibco) supplemented with a serine-free MEM amino acid
922 mixture and MEM vitamins (Gibco), followed by 4 or 18 hours labeling with 2 µl/well

923 L-[^{3}H(G)]serine (30.9 Ci/mmol, NET24800, Perkin-Elmer). After the labeling, the cells
924 were scraped into 0.9 ml 2% NaCl per well, a 0.1 ml aliquot was withdrawn for
925 protein analysis with the BCA assay (Thermo Fisher Scientific), and, after adding 50
926 nmol of unlabeled PS as carrier, the remaining 0.8 ml was subjected to lipid
927 extraction by an acid modification of the Folch method (Kim, Song et al., 2017). After
928 drying, the lipids were resolved in 50 μl $CHCl_3$ and applied on Merck TLC Silica gel
929 60™ plates, followed by separation by using $CHCl_3$-methanol-acetic acid-H2O
930 (50:30:8:3.5) as solvent. The PS and PE spots identified from the mobility of
931 standards run on the same plates were scraped into scintillation vials for analysis of
932 [^{3}H] radioactivity. The DPM values were corrected for total cell protein, and the ratio
933 of [^{3}H] in PE vs. PS calculated.
934
935 **Fluorescence Microscopy**
936 ***Immunofluorescence and Confocal Microscopy***
937 HeLa cells were seeded on 13 mm glass bottom coverslips (Agar Scientific).
938 Immunofluorescence was carried out one day after transfection. Transfected cells
939 were fixed with 4% PFA/PBS for 15 min at room temperature, washed in PBS and
940 incubated with 50 mM NH4Cl/PBS for 15 min at room temperature. After washing
941 with PBS and blocking buffer (1% BSA/ 0,1% Saponin in PBS), cells were incubated
942 with primary antibodies diluted in blocking buffer for 1 hour at room temperature and
943 then with fluorescently-labeled secondary antibodies. After washing with blocking
944 buffer and then PBS, coverslips were mounted on microscopy slides and images
945 were acquired on Confocal inverted microscope SP8-X (DMI 6000 Leica). Optical
946 sections were acquired with a 63x/1.4 Oil immersion objective using the LAS-X
947 software and fluorescent pictures were collected with a PMTs GaAsP hybride camera

948 (Hamamatsu). Images from a mid-focal plane are shown. Images were processed
949 using Image J.
950
951 **Calcium (Ca^{2+}) indicators**

952 ***Ca^{2+} intake assay using Geco constructs***

953 HeLa cells were seeded on glass bottom ibidi chambers (µ-slide 2 wells) 3 days
954 before imaging. Knockdown of the proteins of interest was realized 2 days before
955 imaging, as described above. The day after knockdown, 0.8 µg of GECO plasmid
956 were transfected with 6 µl of lipofectamine 2000/well (Invitrogen), according to the
957 manufacturer's instructions. The day of imaging, cells were washed 4-5 hours before
958 the imaging with fresh DMEM. Cell imaging was performed on an inverted Nikon Ti
959 Eclipse E microscope coupled with a Spinning Disk (Yokogawa CSU-X1-A1) and
960 cage incubator to control both temperature and CO_2 (37 °C, 5% CO2). After
961 excitation with a 561 nm laser (Coherent, 100 mW), fluorescence from GECO
962 constructs was detected with a 40X oil immersion objective (PLAN FLUOR; NA: 1.30;
963 Nikon) and a Prime 95B sCMOS camera (Photometrics). Images were captured
964 every 5 sec during 10 min. Approximatively 1 min after the start of captures,
965 histamine was added to a final concentration of 100 µM to trigger Ca^{2+} release from
966 intracellular stores. Image analysis was performed using Image J by measuring the
967 fluorescence intensity over time (time-lapse) of cells expressing the GECO
968 constructs. Histograms represent the response of the cell to the addition of histamine
969 shown as the maximum fluorescence (Fmax) after treatment / mean fluorescence
970 before histamine F0.

971 ***Ratiometric Ca^{2+} imaging using a mt-Pericam construct***

972 HeLa cells were seeded on glass bottom ibidi chambers (µ-slide 2 wells) 3 days
973 before imaging. Knockdown of the proteins of interest was realized 2 days before
974 imaging, as described above. The day after knockdown, 2.4 µg of mt-Pericam
975 plasmid were transfected with 6 µl of lipofectamine 2000/well (Invitrogen), according
976 to the manufacturer's instructions. The day of imaging, cells were washed 4-5 hours
977 before the imaging with fresh DMEM.
978 Imaging of mt-pericam was performed on an inverted Nikon Ti Eclipse Eclipse-E
979 microscope coupled with a Spinning Disk (Yokogawa, CSU-X1-A1), a 40x CFI Plan
980 Fluor objective (Nikon, NA 1.30, oil immersion) and a sCMOS camera (Photometrics,
981 Prime 95B) with a binning of 2. Knock-down cells expressing pericam constructs
982 were imaged in a controlled atmosphere (37°C, 5% CO_2). For each field of view, a
983 time-lapse was recorded with an image every 8 sec during 10 min. Approximatively 3
984 min after the start of captures, histamine was added to a final concentration of 100
985 µM. For each time point mt-pericam fluorescence was collected using a 525/45
986 bandpass filter (Semrock) after alternative excitation at 405 nm (Vortran, 100mW
987 laser) and 488 nm (Vortran, 150mW laser). A perfect focus system (Nikon) was used
988 to keep the focus constant along the timelapse, the whole system was driven by
989 Metamorph (version 7.7, Molecular Devices).
990 All the image processing and quantification was performed using a custom script in
991 Image J. First, images were corrected for flat-field and dark noise using reference
992 images respectively acquired on a fluorescent plastic slide (Chroma) and with no light
993 reaching the detector. Regions of interest were also drawn on an empty area of every
994 stack to perform a background subtraction on every image. Then hyperstacks
995 containing two channels were registered to correct any lateral drift of the sample
996 during the acquisition using the Image J plugin "HyperStackReg (v05)" with the

997 “translation” algorithm. Once the stacks were processed, regions of interest were
998 drawn around individual cells, a threshold applied (Mean) and the mean intensity of
999 each cell recorded on each image pair along the time-lapse. For every cell the ratio
1000 488/405 was plotted along the time and only the cells with a stable ratio during the
1001 first 3 min time points and with a sharp increase observed after addition of histamine
1002 were kept for the final analysis.
1003
1004 **Flow cytometry for TMRE analysis**
1005 ***Membrane potential studies using TMRE- Mitochondrial Membrane Potential***
1006 ***Assay Kit***
1007 HeLa cells were seeded on 6-well plates 3 days before TMRE detection and the day
1008 after they were transfected for 48 hours with the non-targeting, ORP5 or ORP8-
1009 specific siRNAs specified above. The day before analysis, the medium was replaced
1010 with DMEM 10% FBS with or without 20 μM CCCP (Carbonyl cyanide m-
1011 chlorophenyl hydrazine) at 37°C and 5% CO_2 to induce mitochondrial depolarization.
1012 After 24 hours, 200 nM of TMRE in DMEM 10% FBS (Mitochondrial Membrane
1013 Potential Assay Kit, abcam ab113852) was added to cells and replaced after 30 min.
1014 Cells were kept at 37°C and 5% CO_2 for additional 15 min and then trypsinized and
1015 resuspended in DMEM (1-2x105 cells in 500 μl). TMRE was then analysed by flow
1016 cytometry.
1017 ***Flow cytometry***
1018 Multicolor flow cytometry was conducted on Beckman Coulter’s CytoFLEX S
1019 cytometer. Analyses were performed using Beckman Coulter’s Kaluza and CytExpert
1020 software. TMRE (Ex/Em = 549/575) was excited by a 561 nm laser and detected

1021 through a 585/542 nm filter channel. All acquisitions were made on 10 000 cells. All
1022 data are presented as mean ±SEM of three independent experimental replicates.
1023
1024 **Apoptosis assay using Cleaved-caspase-3 expression**
1025 HeLa cells were seeded on glass bottom 13 mm coverslips in a 24 well plates. The
1026 next day, 48 hours knockdown of the proteins of interest were realized as described
1027 above. 2 days after the knockdown, cells were treated with 1 µM staurosporine or
1028 with DMSO to induce apoptosis. After 4 hours apoptotic cells were analyzed by
1029 confocal immunofluorescence, using cleaved Caspase-3 antibody and Mitotracker
1030 red to label mitochondria. Cleaved Caspase-3 positive cells were quantified using the
1031 Cell Counter plugin from Image J Software. All data are presented as mean ±SEM of
1032 three experimental replicates.
1033
1034 **Senescence studies using SA-β-Galactosidase assay and crystal violet**
1035 **staining**
1036 For SA-β-galactosidase assay, cells were washed twice with PBS and fixed 5 min
1037 with 2% formaldehyde / 0.2% glutaraldehyde. Cells were then rinsed twice in PBS
1038 and incubated at 37°C overnight in SA-β-Gal staining solution as previously
1039 described (Itahana, Campisi et al., 2007).
1040 For crystal violet staining, cells were washed with PBS, fixed for 15 min in 3.7%
1041 formaldehyde and then stained with 0.5% crystal violet solution. After drying, plates
1042 were scanned.
1043 Relative cell count was determined by dissolving crystal violet in acetic acid solution
1044 and measuring optical density at 595 nM.
1045

1046 **Electron Microscopy Analysis**

1047 ***Conventional EM***

1048 For conventional EM, cells grown on 13 mm glass bottom coverslips (Agar Scientific)
1049 were fixed with 2.5% glutaraldehyde and 2% PFA in 0.1 M cacodylate, 0.05% $CaCl_2$
1050 buffer for 24 hours. After several washes with 0.1 M cacodylate buffer, the cells were
1051 postfixed with 1% OsO_4, 1.5% potassium ferricyanide in 0.1M Cacodylate for 1 hour.
1052 After several washes with 0.1 M cacodylate buffer and H_2O, the cells were stained
1053 with 0.5% uranyl acetate for 24 hours. After several washes with H_2O, the cells were
1054 dehydrated in ethanol and embedded in Epon while on the coverslips. Ultrathin
1055 sections were prepared, counterstained with uranyl acetate and observed under a
1056 MET JEOL 1400 equipped with a Orius High speed (Gatan) camera.

1057 ***HRP Detection***

1058 HeLa cells expressing HRP-KDEL were fixed on coverslips with 1.3% glutaraldehyde
1059 in 0.1 M cacodylate buffer, washed in 0.1 M ammonium phosphate [pH 7.4] buffer for
1060 1 hour and HRP was visualized with 0.5 mg/ml DAB and 0.005% H_2O_2 in 0.1 M
1061 Ammonium Phosphate [pH 7.4] buffer. Development of HRP (DAB dark reaction
1062 product) took between 5 min to 20 min and was stopped by extensive washes with
1063 cold water. Cells were postfixed in 2% OsO_4+1% $K_3Fe(CN)_6$ in 0.1 M cacodylate
1064 buffer at 4°C for 1 hour, washed in cold water and then contrasted in 0.5% uranyl
1065 acetate for 2 hours at 4°C, dehydrated in an ethanol series and embedded in epon as
1066 for conventional EM. Ultrathin sections were counterstained with 2% uranyl acetate
1067 and observed under a FEI Tecnai 12 microscope equipped with a CCD (SiS 1kx1k
1068 keenView) camera.

1069 ***Immunogold labelling***

1070 HeLa cells were fixed with a mixture of 2%PFA and 0.125% glutaraldehyde in 0.1 M
1071 phosphate buffer [pH 7.4] for 2 hours, and processed for ultracryomicrotomy as
1072 described previously (Slot & Geuze, 2007). Ultrathin cryosections were single- or
1073 double-immunogold-labeled with antibodies and protein A coupled to 10 or 15 nm
1074 gold (CMC, UMC Utrecht, The Netherlands), as indicated in the legends to the
1075 figures. Immunogold-labeled cryosections were observed under a FEI Tecnai 12
1076 microscope equipped with a CCD (SiS 1kx1k keenView) camera.

1077 ***Quantifications***

1078 For the quantification of the number of cristae junction in Epon sections, about 200
1079 mitochondria were analyzed in randomly selected cell profiles and cristae junctions
1080 were counted in each of the mitochondria profile and reported as number of
1081 cristae/mitochondria profile. All data are presented as mean ±SEM of three
1082 experimental replicates.

1083 For the quantification of ER-mitochondria contact sites in HRP-stained Epon
1084 sections, the total circumference of each mitochondria and the length of the multiple
1085 HRP-positive ER segments closely associated (<30 nm) with them were measured
1086 by iTEM software (Olympus) on acquired micrographs of HeLa cells for each of 20-
1087 30 cell profiles, as indicated in the figure legends. Cells were randomly selected for
1088 analysis without prior knowledge of transfected plasmid or siRNA. All data are
1089 presented as mean (%) ±SEM of three experimental replicates.

1090 For the quantification of immunogold labeling on ultrathin cryosections, 150 gold
1091 particles were counted on acquired micrographs of randomly selected cell profiles at
1092 specific ranges of distance from CJ (0-50, 50-100, 100-150, 150-200 nm) in each of
1093 three experiments. All data are presented as mean (%) ±SEM of three experimental
1094 replicates.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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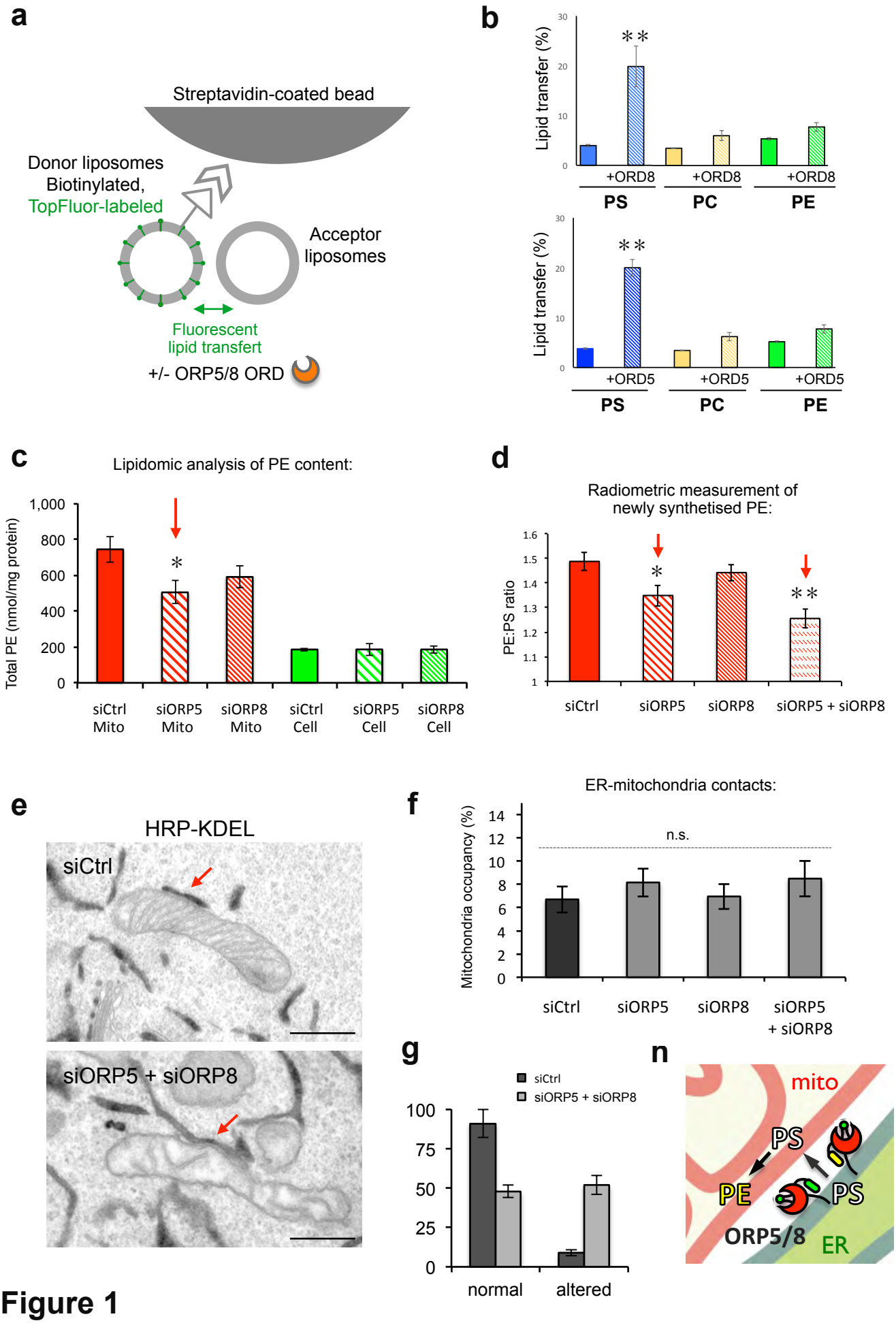

a
Streptavidin-coated bead
Donor liposomes
Biotinylated,
TopFluor-labeled
Acceptor
liposomes
Fluorescent
lipid transfert
+/- ORP5/8 ORD
b
Lipid transfer (%)
30
20
10
0
**
+ORD8
+ORD8
+ORD8
PS
PC
PE
Lipid transfer (%)
30
20
10
0
**
+ORD5
+ORD5
+ORD5
PS
PC
PE
c
Lipidomic analysis of PE content:
Total PE (nmol/mg protein)
1,000
800
600
400
200
0
*
siCtrl Mito
siORP5 Mito
siORP8 Mito
siCtrl Cell
siORP5 Cell
siORP8 Cell
d
Radiometric measurement of
newly synthetised PE:
PE:PS ratio
1.6
1.5
1.4
1.3
1.2
1.1
1
*
**
siCtrl
siORP5
siORP8
siORP5 + siORP8
e
HRP-KDEL
siCtrl
siORP5 + siORP8
f
ER-mitochondria contacts:
Mitochondria occupancy (%)
14
12
10
8
6
4
2
0
n.s.
siCtrl
siORP5
siORP8
siORP5
+ siORP8
g
siCtrl
siORP5 + siORP8
100
75
50
25
0
normal
altered
n
mito
PS
PE
PS
ORP5/8
ER

Figure 1

Figure 1. ORP5 and ORP8 mediate PS transfer *in vitro* and at ER-mitochondria contact sites

(a) Schematic cartoon showing the *in vitro* assay used to study ORP5/8 ORD-mediated lipid transport between liposomes. (b) Donor liposomes containing fluorescent lipids (97 mol% POPC, 1 mol% TopFluor-PS, -PC or -PE and 2 mol% of biotinylated-PE) and pre-bound to streptavidin beads were mixed at a 1:1 molar ratio with acceptor liposomes (100 mol% POPC) in the presence or absence of ORP5 or ORP8 ORD domains (250 µM of donor and acceptor liposomes and 0,3 µM of proteins in the reaction solution). The increase in fluorescence in the acceptor liposomes, which remain unbound in the solution, was measured after 1 hour of incubation at 37°C. Data are presented as % of transferred lipid ±SEM and are the mean of six independent replicates. **P<0,001 (c) Mass spectrometry (MS)-based quantification of the PE content (nmol/mg protein) of mitochondria isolated from Ctrl, ORP5 or ORP8 knockdown cells and of Ctrl, ORP5 or ORP8 knockdown intact cells. Data are shown as mean of three independent replicates ±SEM. *P<0,05. (d) Ctrl, ORP5, ORP8 or ORP5+ORP8 double-knockdown cells were incubated with L-[^{3}H(G)]serine (30.9 Ci/mmol) for 18 hour. After extraction and separation of lipids by TLC, PS and PE spots were scraped and analysed for [^{3}H] radioactivity, as described under “Methods”. Each condition was analyzed in triplicate in each of three independent biological replicates. Data are presented as mean of PE:PS ratio ±SEM. *P<0,05, **P<0,01 compared to Ctrl. (e) Electron micrographs of HRP-KDEL-expressing HeLa cells treated with Ctrl siRNAs or siRNAs against ORP5 and ORP8 (siORP5+siORP8). Red arrows indicate ER-mitochondria contact sites. Scale bar, 500 nm. (f) Quantifications of the extent of ER-mitochondria contact sites in Ctrl, ORP5, ORP8 and ORP5+ORP8 knockdown cells expressing HRP-KDEL. Data are shown as % of the ER in contact with mitochondria (mitochondria occupancy) ±SEM, n = 20 cell profiles and ±900 mitochondria; n.s; not significant. (g) Quantifications of the number of mitochondria with aberrant cristae morphology in the indicated siRNA conditions. Data are shown as % of mitochondria ±SEM, n = 20 cell profiles and ±700 mitochondria. **P<0,01 compared to siCtrl. (h) Schematic representation of non-vesicular PS transfer mediated by ORP5/8 at ER-mitochondria contact sites. PS is transported to mitochondrial membranes where it is rapidly converted into mitochondrial PE.

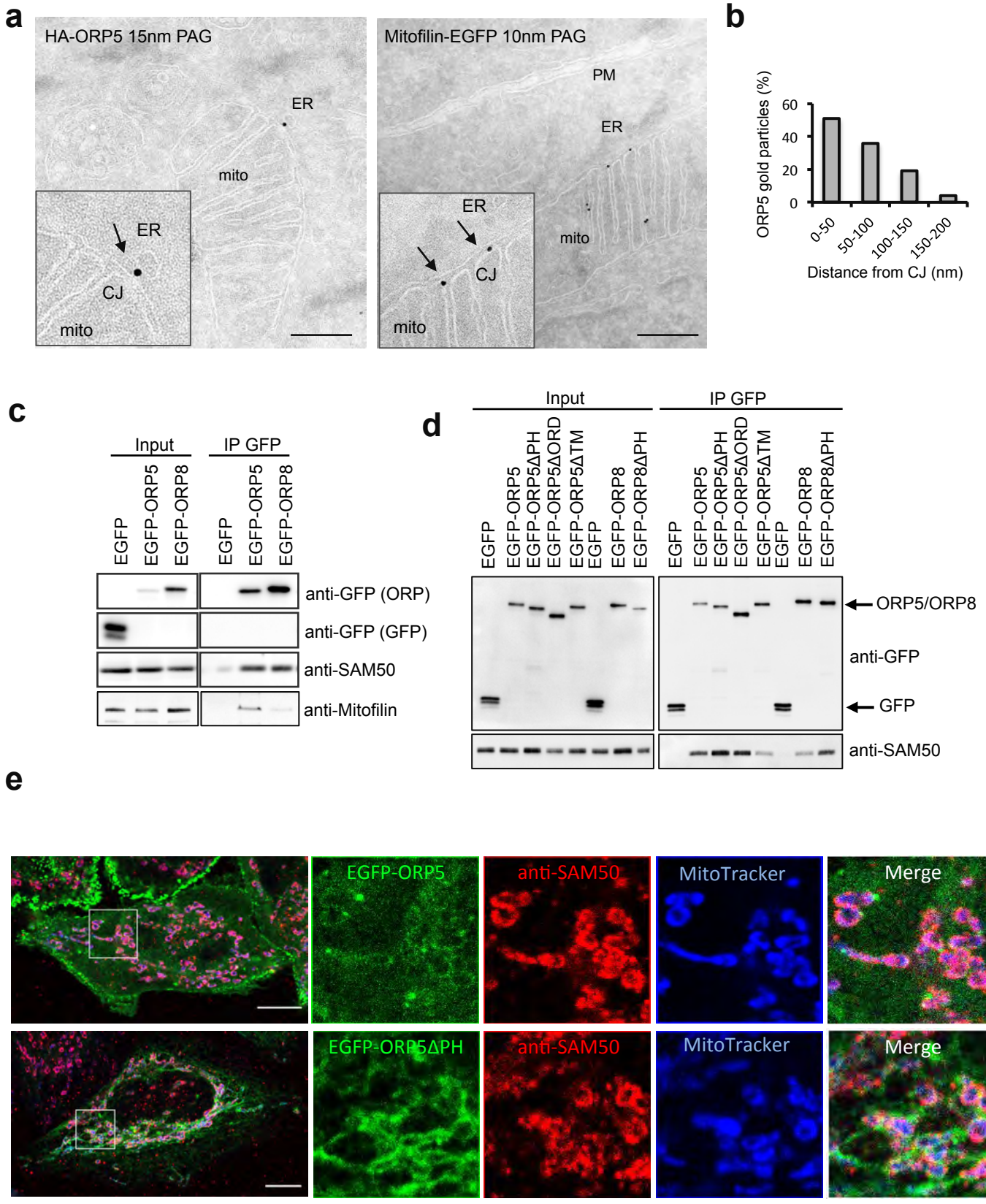

a
HA-ORP5 15nm PAG
ER
mito
ER
CJ
mito
Mitofilin-EGFP 10nm PAG
PM
ER
mito
ER
CJ
mito
b
ORP5 gold particles (%)
60
40
20
0
0-50
50-100
100-150
150-200
Distance from CJ (nm)
c
Input
IP GFP
EGFP
EGFP-ORP5
EGFP-ORP8
anti-GFP (ORP)
anti-GFP (GFP)
anti-SAM50
anti-Mitofilin
d
Input
IP GFP
EGFP
EGFP-ORP5
EGFP-ORP5ΔPH
EGFP-ORP5ΔORD
EGFP-ORP5ΔTM
EGFP
EGFP-ORP8
EGFP-ORP8ΔPH
ORP5/ORP8
anti-GFP
GFP
anti-SAM50
e
EGFP-ORP5
anti-SAM50
MitoTracker
Merge
EGFP-ORP5ΔPH
anti-SAM50
MitoTracker
Merge

Figure 2

Figure 2. ORP5 and ORP8 interact with the MIB/MICOS complex at ER-mitochondria contacts near CJ

(a) Electron micrograph of ultrathin cryosection of HeLa cells transfected with HA-ORP5 or Mitofilin-EGFP and immunogold stained with anti-HA or anti-GFP (10 or 15 nm gold) showing HA-ORP5 localization at ER-mitochondria contacts in close proximity to CJ (arrow) and the localization of the MICOS complex (Mitofilin) at CJ (arrows). Scale bar 150 nm. (b) Quantification of the proximity of HA-ORP5 gold particles to the CJ. Results are presented as the percentage of ORP5 gold particles (n = 23 cell profiles) at specific ranges of distance from CJ (0-50, 50-100, 100-150, 150-200 nm). (c) EGFP-ORP5, EGFP-ORP8 or EGFP alone were transfected in HeLa cells then immuno-precipitated from lysates and analyzed by western blot using antibodies against GFP (for ORP5 or ORP8), Mitofilin or SAM50. (d) EGFP-ORP5, EGFP-ORP5ΔPH, EGFP-ORP5ΔORD, EGFP-ORP5ΔTM, EGFP-ORP8, EGFP-ORP8ΔPH or EGFP alone were transfected in HeLa cells then immuno-precipitated from lysates and analyzed by western blot using antibodies against GFP (for ORP5 or ORP8) or against SAM50. (e) Fluorescent images of HeLa cells transfected with either EGFP-ORP5 or EGFP-ORP5ΔPH and stained with MitoTracker and anti-SAM50 antibody. Insets show magnifications of the boxed regions. Scale bar, 10 μm.

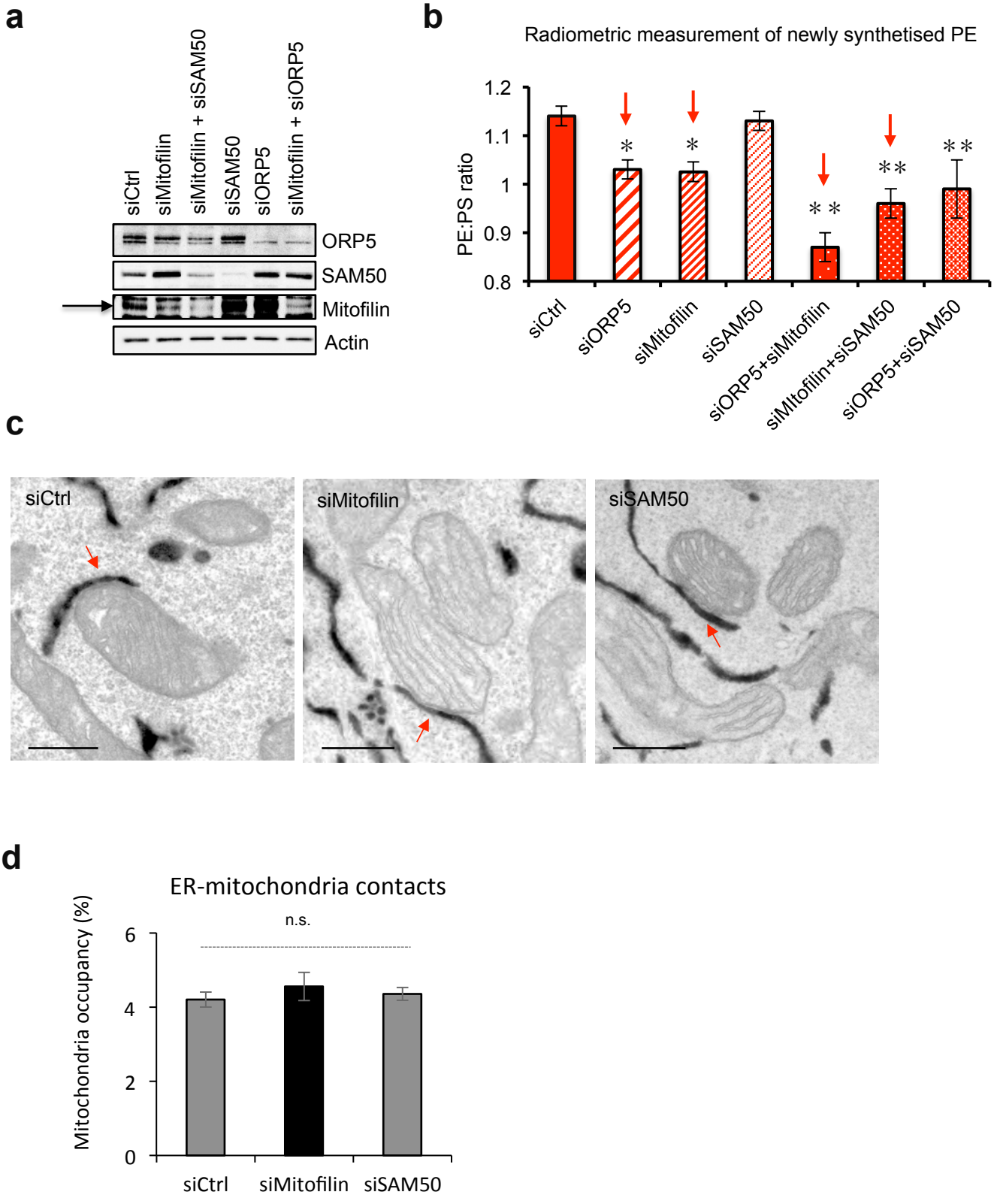
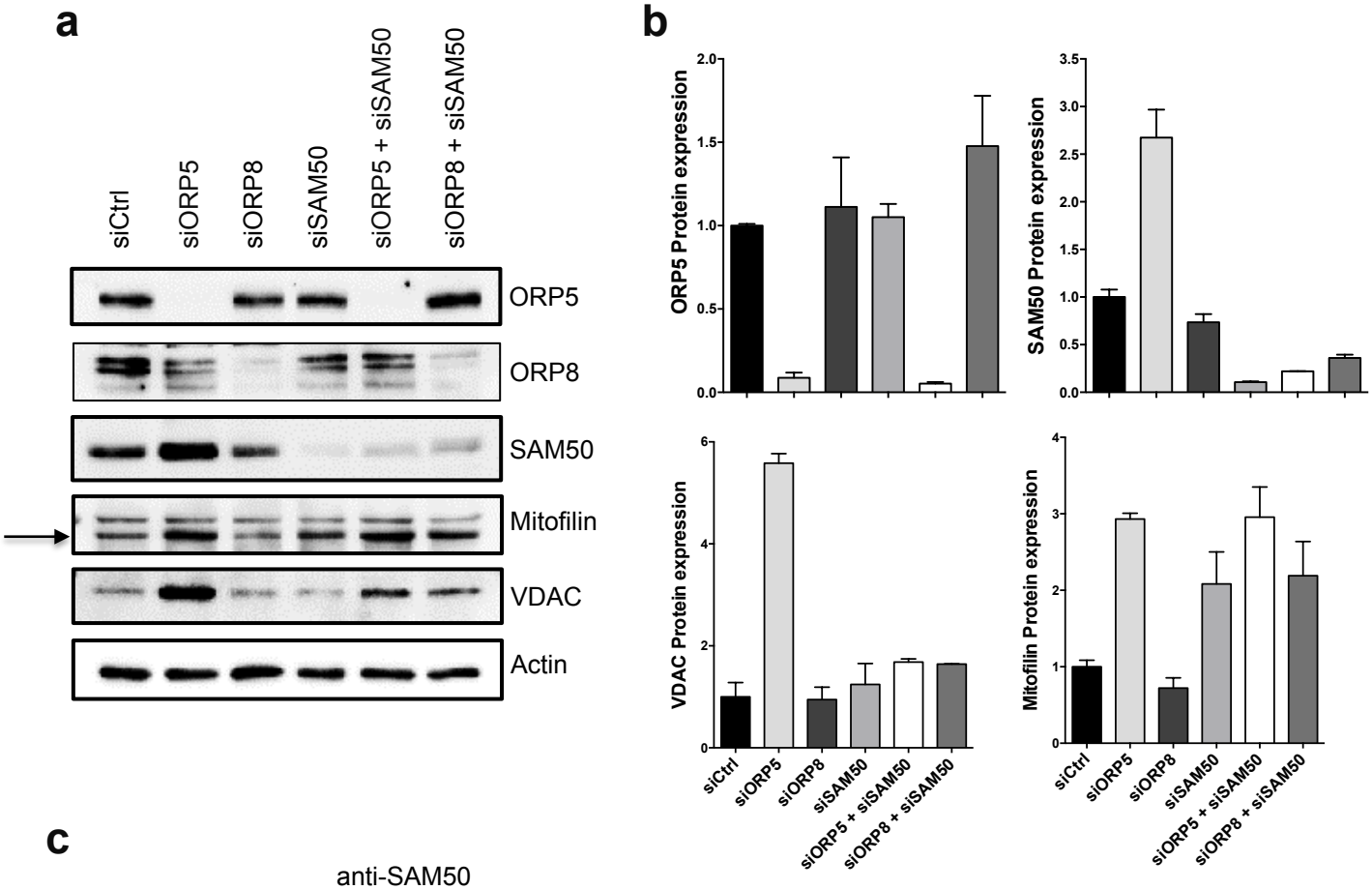
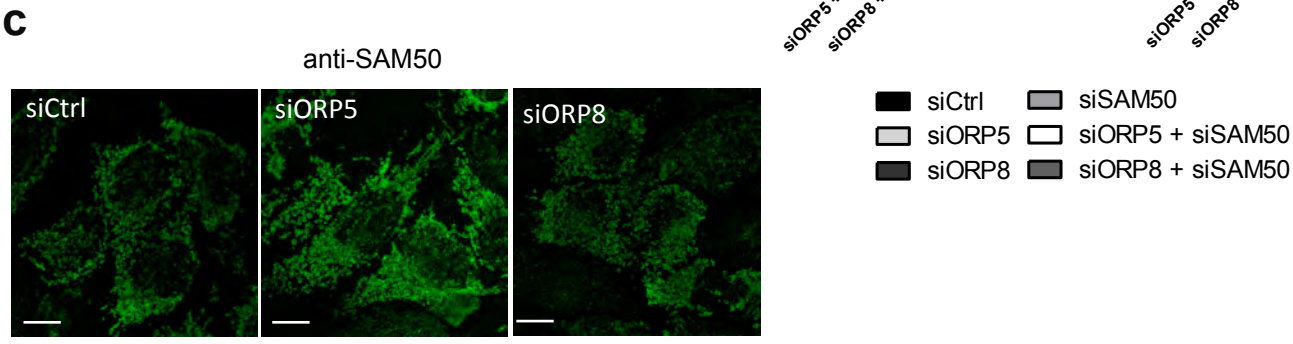

a
siCtrl
siMitofilin
siMitofilin + siSAM50
siSAM50
siORP5
siMitofilin + siORP5
ORP5
SAM50
Mitofilin
Actin
b
Radiometric measurement of newly synthetised PE
PE:PS ratio
1.2
1.1
1
0.9
0.8
siCtrl
siORP5
siMitofilin
siSAM50
siORP5+siMitofilin
siMltofilin+siSAM50
siORP5+siSAM50
c
siCtrl
siMitofilin
siSAM50
d
ER-mitochondria contacts
n.s.
Mitochondria occupancy (%)
6
4
2
0
siCtrl
siMitofilin
siSAM50

Figure 3

Figure 3. ORP5 and the MIB/MICOS complex cooperate to mediate non-vesicular transport of PS from the ER to mitochondrial membranes

(a) Western analysis showing ORP5, SAM50, Mitofilin and Actin levels in protein lysates from HeLa cells treated with siRNA against Ctrl, ORP5, Mitofilin or SAM50. Arrow indicates the specific band for Mitofilin. (b) Radiometric measurement of PS-to-PE conversion in the indicated siRNAs. Data are presented as mean of PE:PS ratio ±SEM. Each condition was performed in triplicate in each of the independent biological replicates (n = 5 for siCtrl and siORP5; n = 4 for siSAM50; n = 3 for the other siRNAs conditions). *P<0,05, **P<0,01 compared to Ctrl. (c) Representative electron micrographs of HeLa cells treated with Ctrl siRNAs or siRNAs against Mitofilin or SAM50 and transfected with HRP-KDEL. Red arrows indicate ER-mitochondria contact sites. Scale bar, 500 nm. (d) Quantifications of the extent of ER-mitochondria contact sites in Ctrl, Mitofilin and SAM50 knockdown cells expressing HRP-KDEL. Data are shown as % of the ER in contact with mitochondria (mitochondria occupancy) ±SEM, n = 30 for siCtrl, n = 20 cell profiles for siMitofilin and siSAM50 and 1 000 mitochondria; n.s; not significant.


a
siCtrl
siORP5
siORP8
siSAM50
siORP5 + siSAM50
siORP8 + siSAM50
ORP5
ORP8
SAM50
Mitofilin
VDAC
Actin
b
ORP5 Protein expression
SAM50 Protein expression
VDAC Protein expression
Mitofilin Protein expression
siCtrl
siORP5
siORP8
siSAM50
siORP5 + siSAM50
siORP8 + siSAM50


c
anti-SAM50
siCtrl
siORP5
siORP8
siCtrl
siORP5
siORP8
siSAM50
siORP5 + siSAM50
siORP8 + siSAM50

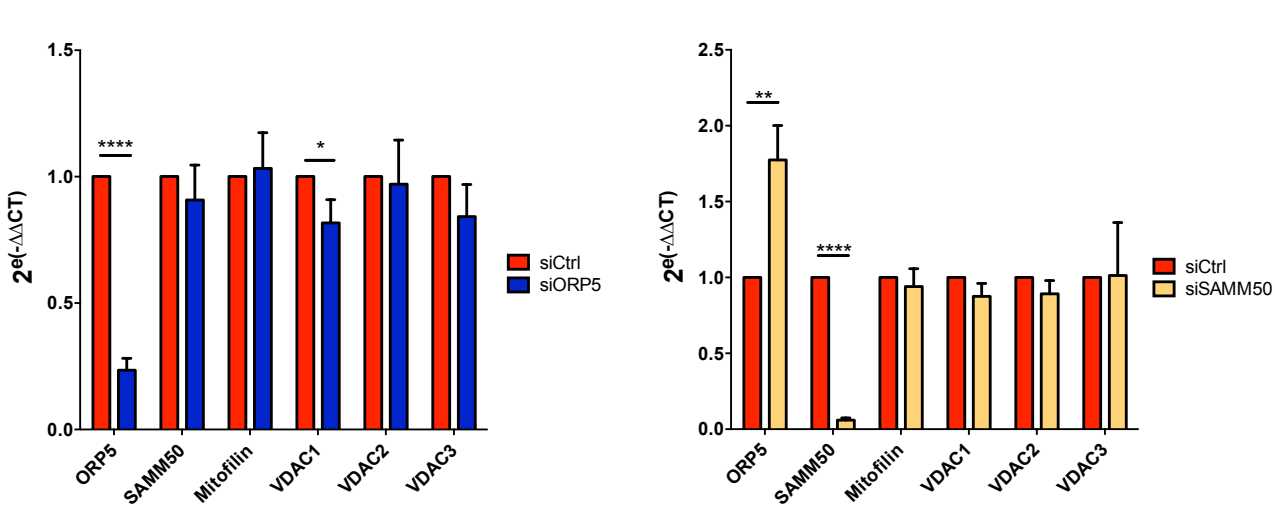

d
2e(-ΔΔCT)
****
*
siCtrl
siORP5
ORP5
SAMM50
Mitofilin
VDAC1
VDAC2
VDAC3
**
****
siCtrl
siSAMM50

Figure 4

Figure 4. Silencing of ORP5 but not ORP8 specifically increases VDAC protein level in a SAM50-dependent manner

(a) Western analysis showing ORP5, ORP8, SAM50, Mitofilin, VDAC and Actin levels in protein lysates from HeLa cells treated with either Ctrl siRNAs, or with siRNAs against ORP5, ORP8, or SAM50. (b) Quantification of ORP5, SAM50, VDAC and Mitofilin protein expression in the indicated knockdown conditions. Normalized data are shown as mean ±SEM (n = 3). (c) Confocal images of Ctrl, ORP5 and ORP8 knockdown HeLa cells immunostained using anti-SAM50 antibody. Scale bar, 10 µm. (d) Quantitative RT-PCR analysis of ORP5, SAM50, Mitofilin, VDAC1, VDAC2 and VDAC3 in ORP5 (left graph) or in SAM50 (right graph) knockdown cells versus control HeLa cells. y axis: $2^{(-\Delta\Delta Ct)}$ value represents differences between the mean Ct (Cycle threshold) values of tested genes and those of reference gene (SDHA).

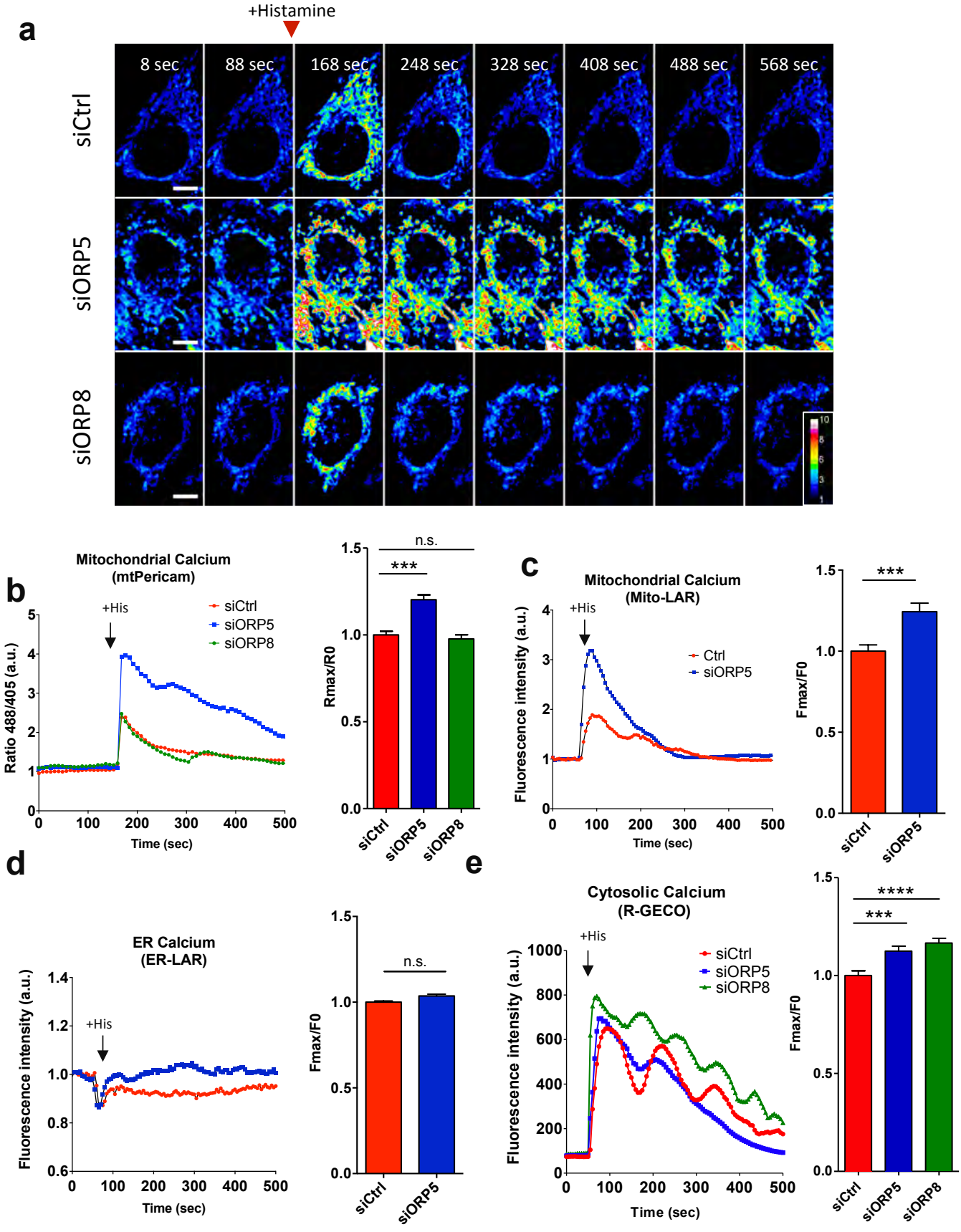

a
+Histamine
8 sec
88 sec
168 sec
248 sec
328 sec
408 sec
488 sec
568 sec
siCtrl
siORP5
siORP8
b
Mitochondrial Calcium
(mtPericam)
+His
siCtrl
siORP5
siORP8
Ratio 488/405 (a.u.)
Time (sec)
Rmax/R0
n.s.
***
c
Mitochondrial Calcium
(Mito-LAR)
Ctrl
siORP5
Fluorescence intensity (a.u.)
Fmax/F0
d
ER Calcium
(ER-LAR)
e
Cytosolic Calcium
(R-GECO)
****

Figure 5

Figure 5. Silencing of ORP5 but not ORP8 increases mitochondrial calcium

(a) Pseudocolor 488/405 ratio time-lapse images of a representative Ctrl, ORP5 or ORP8 knockdown HeLa cell expressing mt-Pericam before and after histamine treatment. Scale bar 10 μm. (b) Representative curves of Ctrl, ORP5 and ORP8 knockdown HeLa cells expressing mt-Pericam showing the ratio of Pericam fluorescence before and after histamine treatment (+His). The change in the ratio of Pericam fluorescence after histamine treatment under each condition was compared (Rmax/R0). Data are presented as normalized mean ±SEM, n = 3 per condition, ***, $p < 0.001$; n.s; not significant. (c) Representative curves of Ctrl and ORP5 knockdown HeLa cells expressing the GECO construct Mito-LAR showing the fluorescence before and after histamine treatment. The change in fluorescence after histamine treatment under each condition was compared (Fmax/F0). Data are presented as normalized mean ±SEM, n = 3 per condition, ***, $p < 0.001$; n.s; not significant. (d) Representative curves of Ctrl and ORP5 knockdown HeLa cells expressing the GECO construct ER-LAR showing the fluorescence before and after histamine treatment. The change in fluorescence after histamine treatment under each condition was compared (Fmax/F0). Data are presented as normalized mean ±SEM, n = 3 per condition, n.s; not significant. (e) Representative curves of Ctrl, ORP5 and ORP8 knockdown HeLa cells expressing the GECO construct R-GECO showing the fluorescence before and after histamine treatment. The change in fluorescence after histamine treatment under each condition was compared (Fmax/F0). Data are presented as normalized mean ±SEM, n = 3 per condition, ***, $p < 0.001$ and ****, $p < 0.0001$; n.s; not significant.

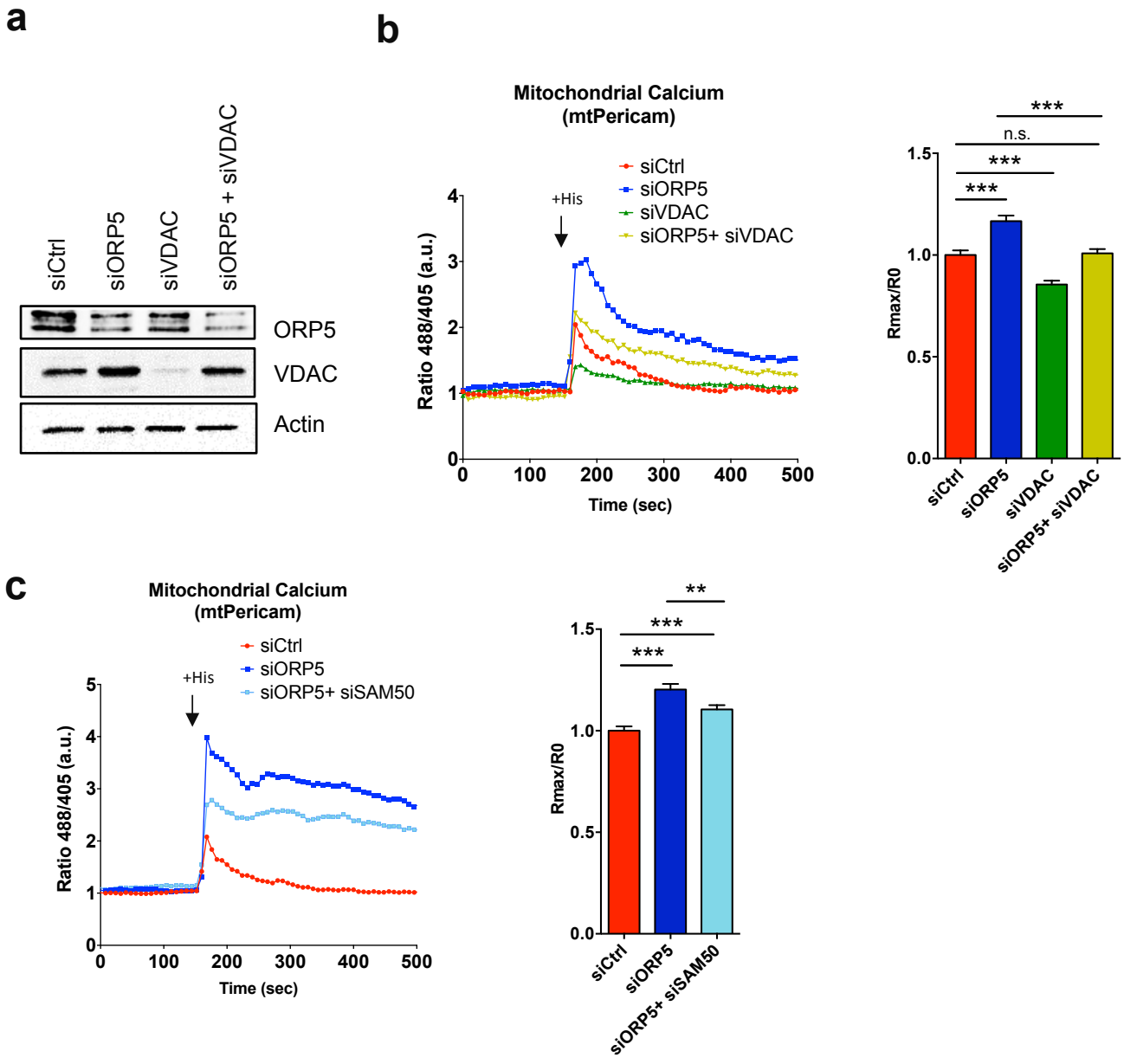
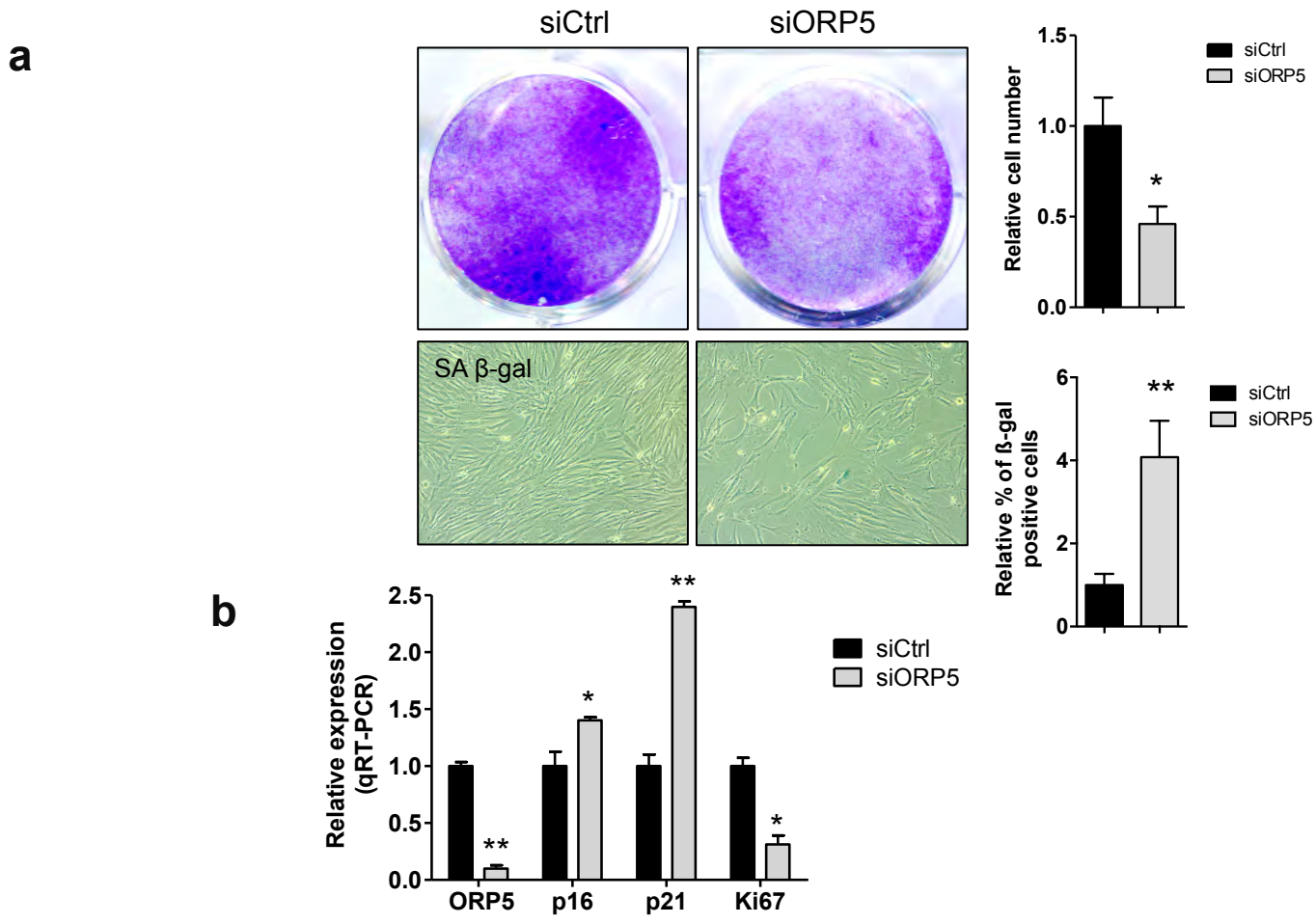

a
siCtrl
siORP5
siVDAC
siORP5 + siVDAC
ORP5
VDAC
Actin
b
Mitochondrial Calcium
(mtPericam)
+His
siCtrl
siORP5
siVDAC
siORP5+ siVDAC
Ratio 488/405 (a.u.)
Time (sec)
0
100
200
300
400
500
0
1
2
3
4
***
n.s.
***
***
1.5
1.0
0.5
0.0
Rmax/R0
siCtrl
siORP5
siVDAC
siORP5+ siVDAC
c
Mitochondrial Calcium
(mtPericam)
+His
siCtrl
siORP5
siORP5+ siSAM50
Ratio 488/405 (a.u.)
Time (sec)
0
100
200
300
400
500
0
1
2
3
4
5
**
***
***
1.5
1.0
0.5
0.0
Rmax/R0
siCtrl
siORP5
siORP5+ siSAM50

Figure 6

Figure 6. Calcium increase induced by ORP5 knockdown is dependent on SAM50 and VDAC

(a) Western analysis showing ORP5 and VDAC protein levels in Ctrl, ORP5 and VDAC siRNA-treated HeLa cells. (b). Representative curves of Ctrl, ORP5, VDAC and ORP5+VDAC knockdown HeLa cells expressing mt-Pericam showing the ratio of Pericam fluorescence before and after histamine treatment (+His). The change in the ratio of Pericam fluorescence after histamine treatment under each condition was compared (Rmax/R0). Data are presented as normalized mean ±SEM, n = 3 per condition, ***, $p < 0.001$; n.s; not significant. (c). Representative curves of Ctrl, ORP5 and ORP5+SAM50 knockdown HeLa cells expressing mt-Pericam showing the ratio of Pericam fluorescence before and after histamine treatment (+His). The change in the ratio of Pericam fluorescence after histamine treatment under each condition was compared (Rmax/R0). Data are presented as normalized mean ±SEM, n = 3 per condition, ***, $p < 0.001$; n.s; not significant.


a
siCtrl
siORP5
SA β-gal
Relative cell number
1.5
1.0
0.5
0.0
*
siCtrl
siORP5
Relative % of ß-gal positive cells
6
4
2
0
**
b
Relative expression (qRT-PCR)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
*
ORP5
p16
p21
Ki67
siCtrl
siORP5

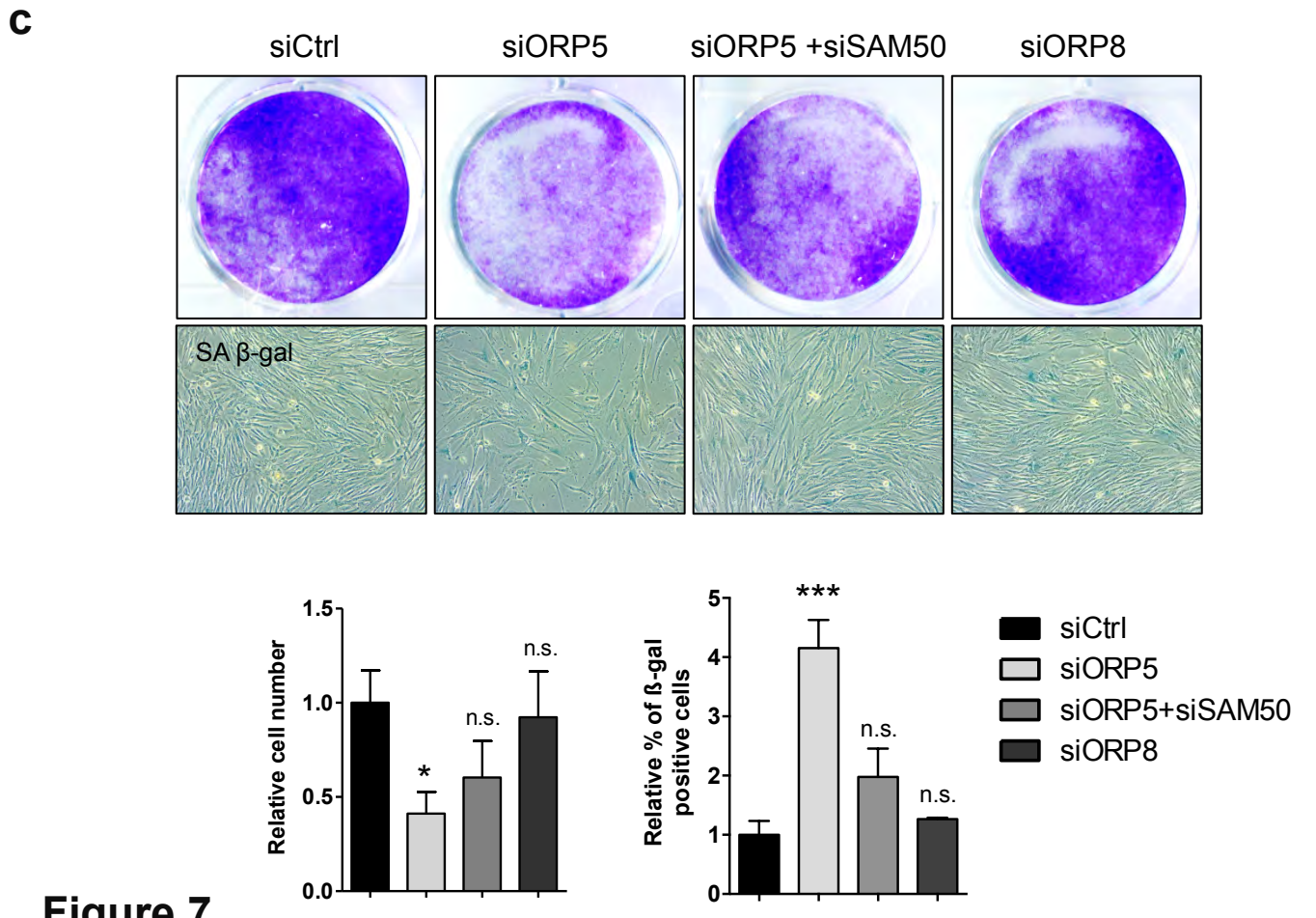

c
siCtrl
siORP5
siORP5 +siSAM50
siORP8
SA β-gal
Relative cell number
1.5
1.0
0.5
0.0
*
n.s.
n.s.
Relative % of ß-gal positive cells
5
4
3
2
1
0
***
n.s.
n.s.
siCtrl
siORP5
siORP5+siSAM50
siORP8

Figure 7

Figure 7. Cell senescence promoted by ORP5 knockdown is dependent on SAM50

(a) MCR5 cells transfected with control siRNA or with siRNA against ORP5 were fixed and then stained with crystal violet solution (CA) or with SA-β-galactosidase (SA-β-Gal) staining solution. The graph on the top right shows the relative cell number determined by crystal violet staining. The graph on the bottom right shows the relative percentage of β-gal positive cells determined by SA-β-galactosidase assay. Data are presented as normalized mean ±SEM, n = 3 per condition, **, $p< 0.01$; *, $p< 0.05$. (b) Quantitative RT-PCR analysis of ORP5, p16, p21 and Ki67 in ORP5 knockdown cells and control HeLa cells. y axis:$2^{(-\Delta\Delta Ct)}$ value represents differences between the mean Ct (Cycle threshold) values of tested genes and those of reference genes (PGK1 and HPRT1). Data are presented as normalized mean ±SEM, n = 3 per condition, **, $p< 0.01$; *, $p< 0.05$. (c) MCR5 cells transfected with control siRNA or with siRNA against ORP5, SAM50 or ORP8 were fixed and then stained with crystal violet solution or with SA-β-Gal staining solution. The graph on the left shows the relative cell number determined by crystal violet staining. The graph on the right shows the relative percentage of β-gal positive cells determined by SA-β-galactosidase assay. Data are presented as normalized mean ±SEM, n = 3 per condition, ***, $p< 0.001$; *, $p< 0.05$; n.s; not significant.

a

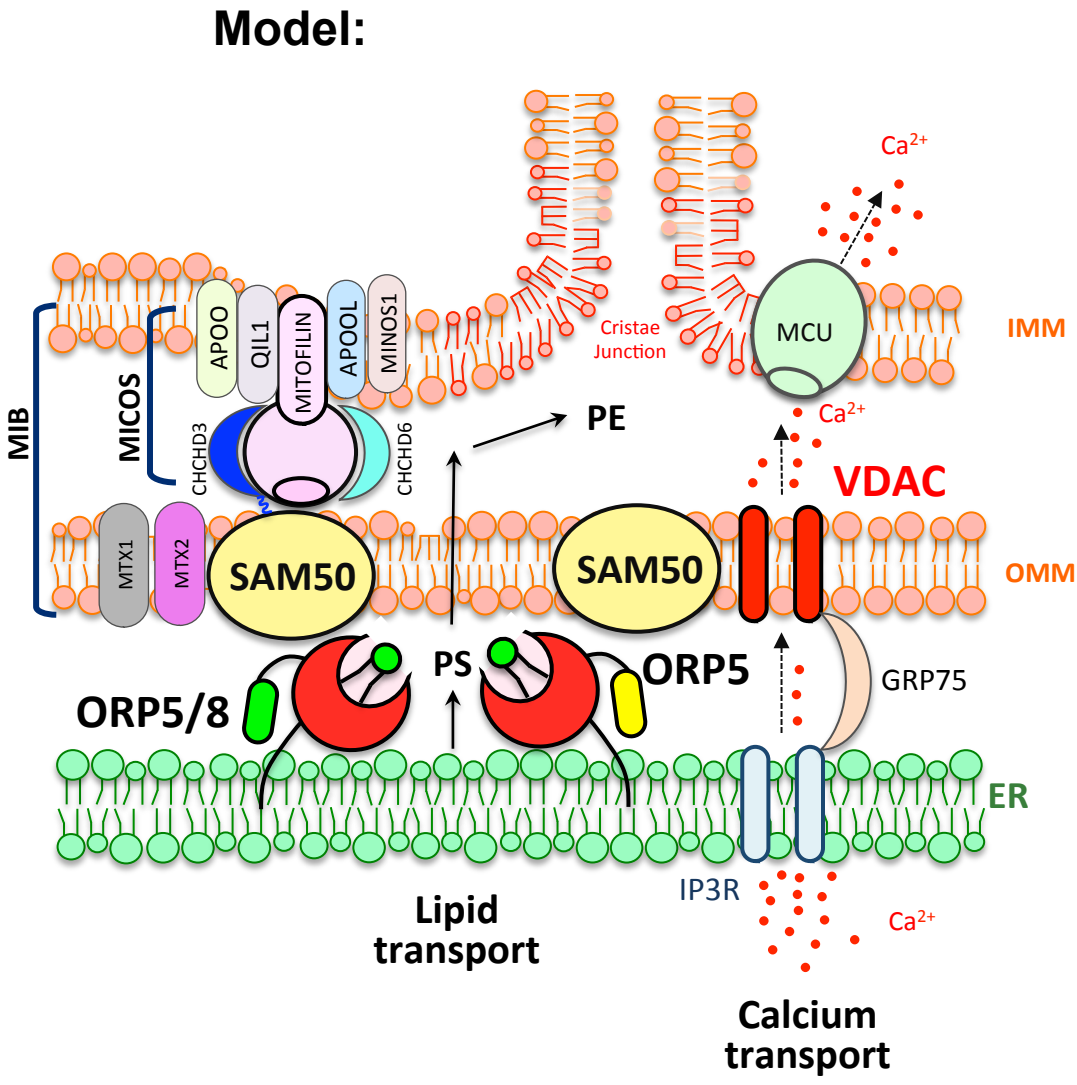


Figure 8. Lipid and calcium transport occur at the same ER-mitochondria contact site microdomains and are regulated by a ORP5-SAM50-VDAC pathway

ORP5/8 are directly involved in the transfer of PS from ER to mitochondria at ER-mitochondria membrane contact sites. This transfer occurs at the level of cristae junctions where ORP5/8 localize and interacts with the proteins of the MIB complex SAM50 and Mitofilin. This interaction facilitates the transfer of PS from ER to IMM at the level of the CJ where the PS is then converted into PE that play a critical role in cristae organization and mitochondrial function. ORP5 is also involved in calcium homeostasis by regulating the level of VDAC *via* SAM50 interaction and regulation.

Figure 8

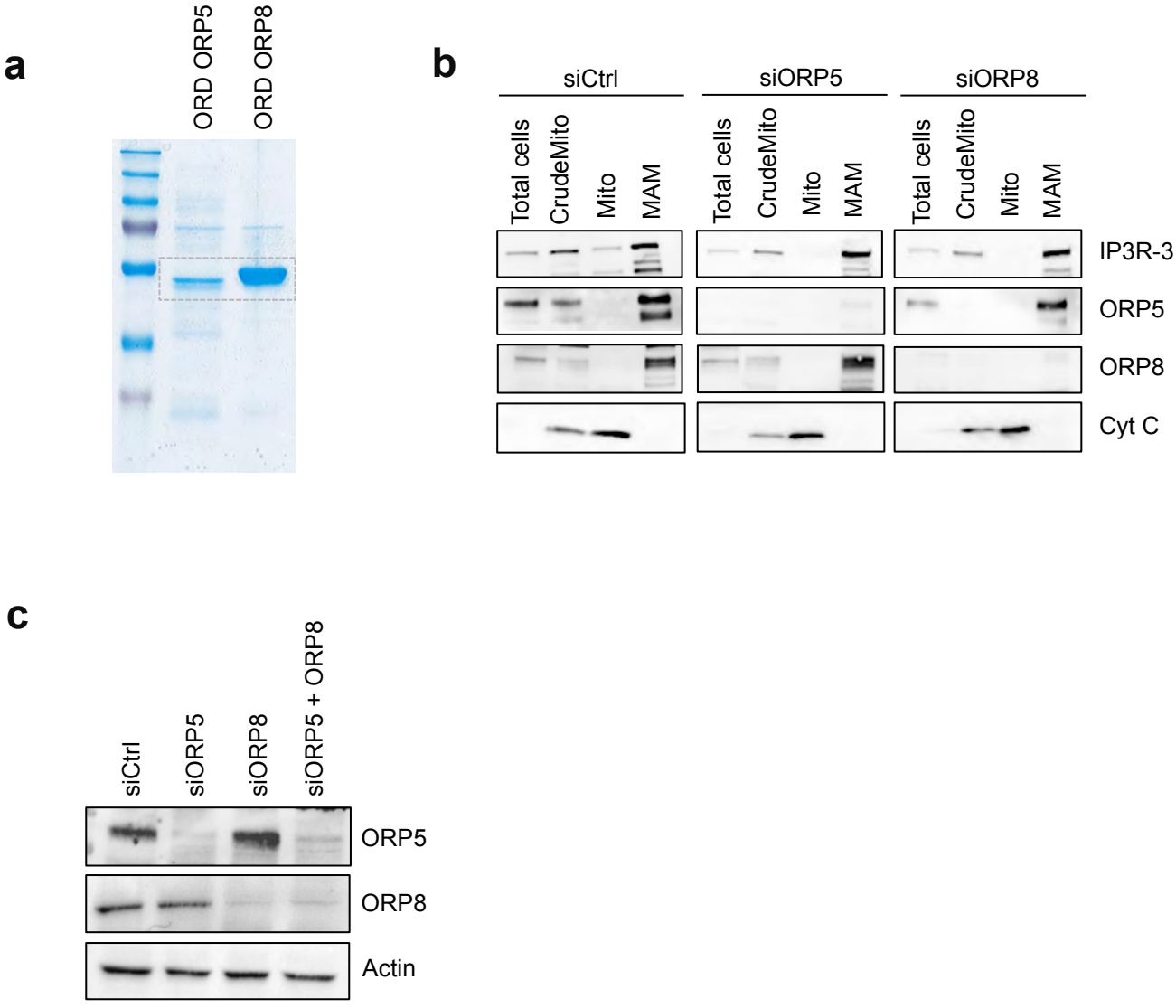


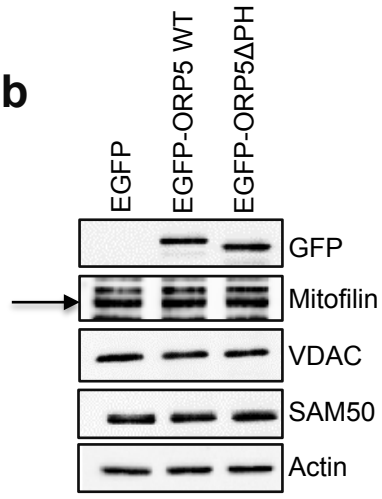
Figure S1. (a) Coomassie stained SDS-PAGE of the recombinant ORD domain of ORP5/8 proteins purified in BL21DE3 RILP cells. (b) Crude mitochondria, mitochondria, and MAM fractions were purified from Ctrl, ORP5 and ORP8 siRNA-treated HeLa cells. Equal amounts of protein from each fraction were loaded on a 4–20% gradient SDS–PAGE gel and immunoblotted using anti-ORP5, anti-ORP8, anti-IP3R-3 (MAM protein), and anti-cytochrome c (mitochondrial protein). Mito, mitochondria; MAM, mitochondria-associated ER membrane. (c) WB analysis showing ORP5, ORP8 and Actin levels in protein lysates from HeLa cells treated with either Ctrl siRNAs or with siRNAs against ORP5 or/and ORP8.

Figure S1

a

		IP EGFP	IP EGFP-ORP5	IP EGFP-ORP5ΔPH
Protein	**Localization**	**Mascot Score**		
ORP5	MAMs	0	9689	12192
ORP8	MAMs	31	930	690
Mitofilin	IMM	43	579	1150
SAM50	OMM	0	237	516
PSS1	MAMs	47	175	167
TOM22	OMM	0	77	95
PTPIP51	OMM	0	76	73
Metaxin2	OMM	0	32	175

b



c

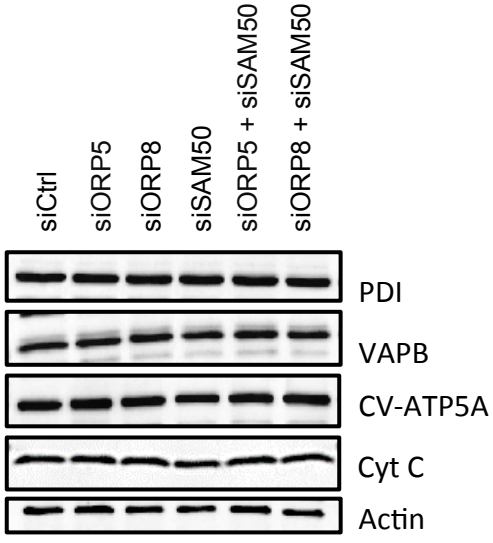


Figure S2. (a) Identification of proteins that interact with EGFP-tagged ORP5 constructs by mass spectrometry. Proteins associated to MAMs and mitochondrial outer and inner membranes (OMM, IMM) are interacting partners of EGFP-ORP5 constructs. Note the presence of some proteins of the MIB complex: Mitofilin, SAM50 and Metaxin2. The interaction of Mitofilin, SAM50 and Metaxin2 with the EGFP-ORP5ΔPH construct is stronger than with EGFP-ORP5. (b) WB analysis showing GFP (EGFP-tagged constructs), Mitofilin, VDAC and SAM50 and Actin levels in protein lysates from HeLa cells transfected with either EGF construct (Control) or with EGFP-ORP5 or EGFP-ORP5ΔPH constructs. (c) WB analysis showing PDI (ER), VAPB (ER), CV-ATP5A (IMM), Cytochrome C (Cyt C, IMM) and Actin levels in protein lysates from HeLa cells treated with either Ctrl siRNAs or with siRNAs against ORP5, ORP8, or SAM50.

Figure S2

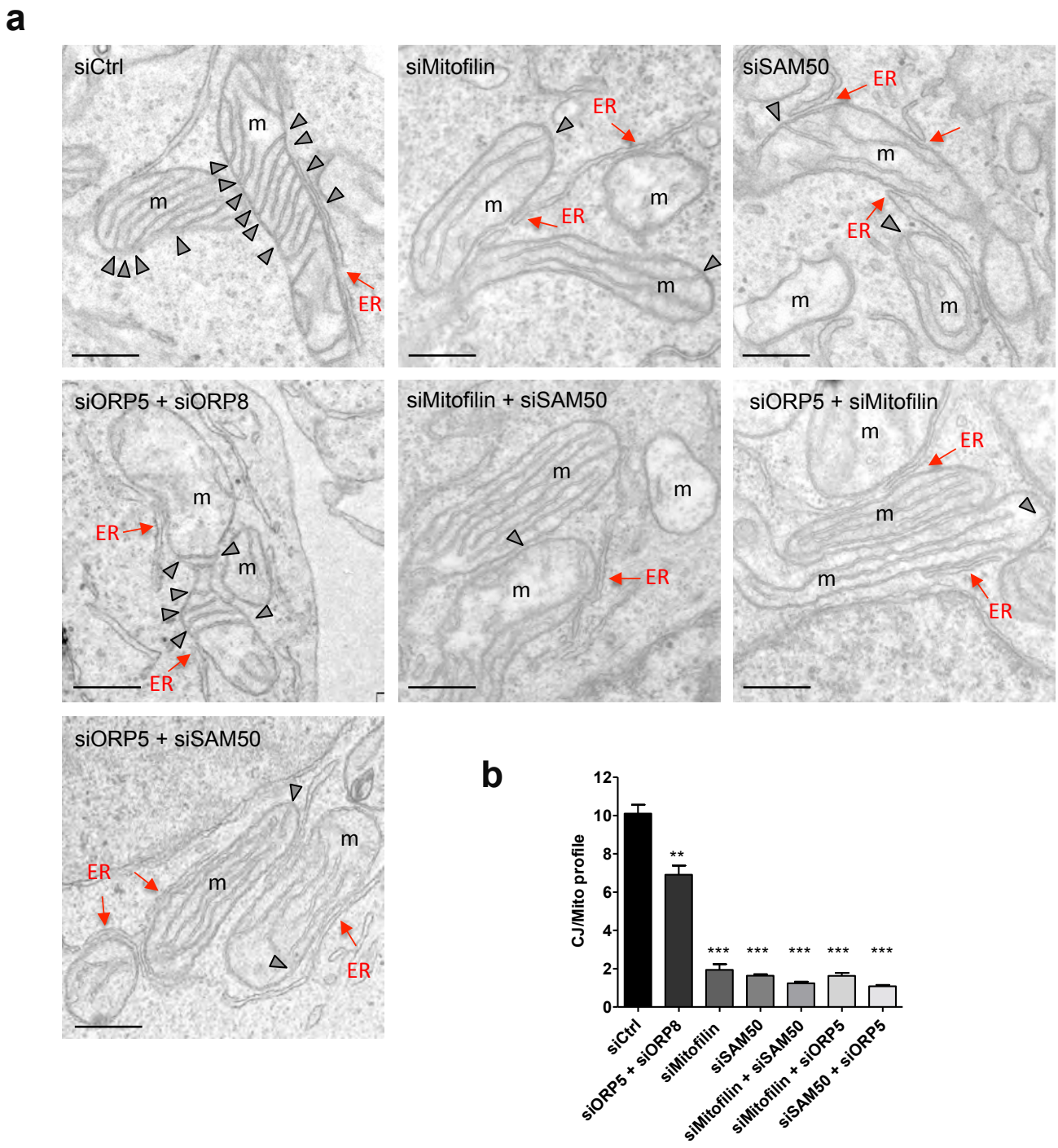


Figure S3. (a) Representative EM micrographs showing the morphology of mitochondria in HeLa cells treated with siRNA against ORP5, ORP8, Mitofilin and SAM50. Scale bar, 200 nm. Red arrows indicate ER elements in contact with mitochondria; arrowheads indicate CJ; m, mitochondria. (b) Quantifications of the number of CJ per mitochondria profile in the indicated siRNA conditions. % of CJ ±SEM, n = 170-260 mitochondria, ***, p< 0.001; **, p< 0.01.

Figure S3

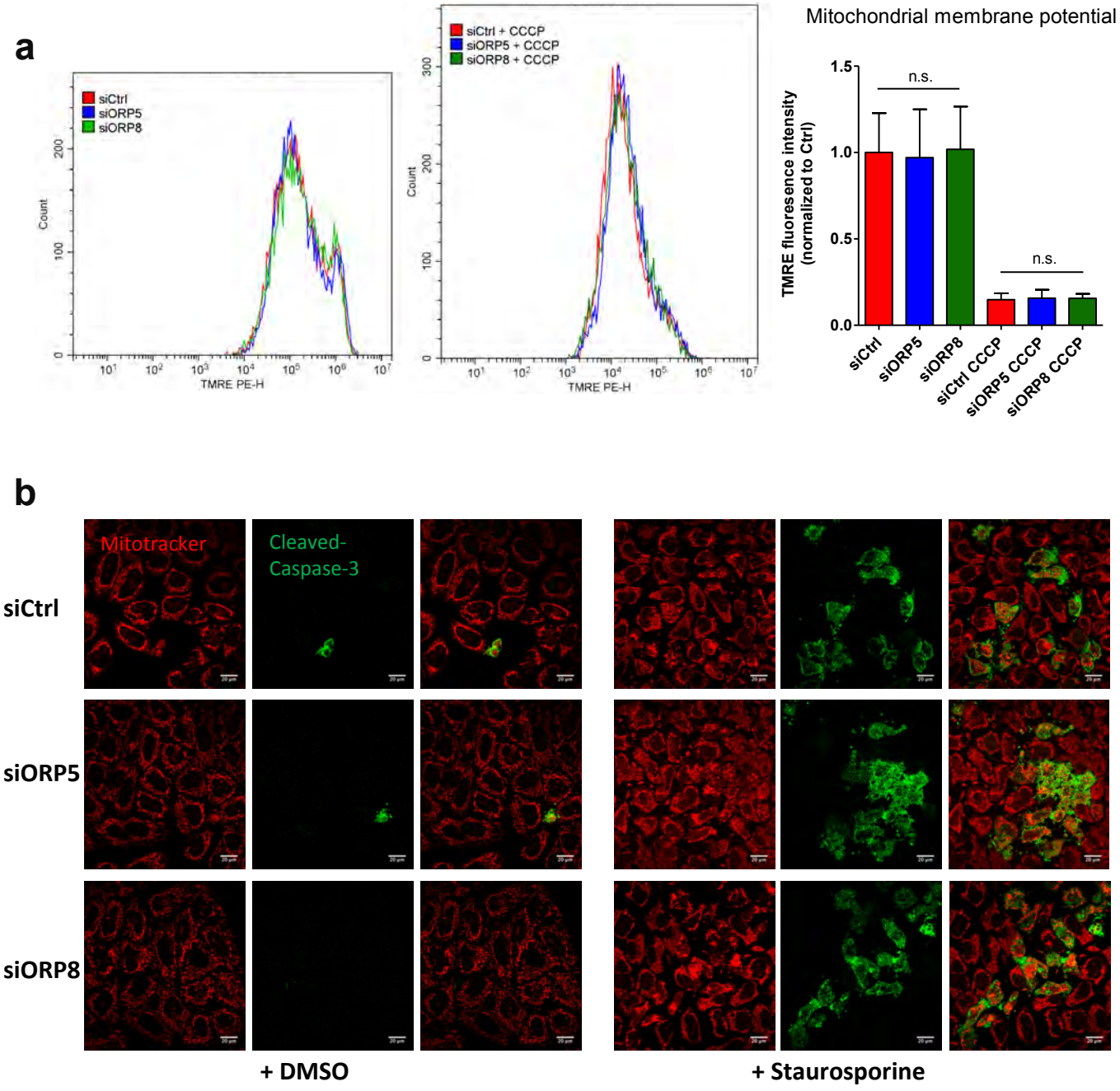

a
siCtrl
siORP5
siORP8
Count
TMRE PE-H
siCtrl + CCCP
siORP5 + CCCP
siORP8 + CCCP
Mitochondrial membrane potential
TMRE fluoresence intensity (normalized to Ctrl)
n.s.
n.s.
siCtrl
siORP5
siORP8
siCtrl CCCP
siORP5 CCCP
siORP8 CCCP
b
Mitotracker
Cleaved-Caspase-3
siCtrl
siORP5
siORP8
+ DMSO
+ Staurosporine
Cleaved Caspase-3 positive cells (% of Total cells)
n.s.
n.s.
siCtrl DMSO
siORP5 DMSO
siORP8 DMSO
siCtrl Stauro
siORP5 Stauro
siORP8 Stauro

Figure S4

Figure S4. (a) Representative graphs of flow cytometry analysis of HeLa cells transfected with siRNA against ORP5 or ORP8 (n = 10 000 cells) and loaded with the mitochondrial membrane potential sensitive dye TMRE, in presence (right graph) or absence (left graph) of CCCP. Flow cytometry histograms are showing the relative TMRE fluorescence intensity; the average of three independent replicates is shown. Data are presented as normalized mean ±SEM. (b) Fluorescent images of HeLa cells transfected with siRNA against ORP5, ORP8 or control, treated with DMSO or staurosporine and stained with anti-cleaved Caspase-3 (apoptotic cells) and Mitotracker. Scale bar, 20 μm. The graphs represent the means ±SEM of the percentage of cleaved Caspase-3 positive cells in the different conditions; the average of three independent replicates is shown, n.s; not significant.